BOOTH, II, WILLIAM T., M.S. Characterization of the Biochemical Activity of the Open Reading Frame "*yqiQ*" of *Bacillus Subtilis.* (2011)
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The goal of this research is to provide a greater chemical understanding of the mechanisms by which *Bacillus subtilis* transforms itself into a spore during times of nutrient deprivation. The area of interest of this study lies in the mother cell metabolic gene (*mmg*) operon, which is activated by a sigma-E transcription factor, during the early stages of sporulation. This operon is proposed to play roles in fatty acid metabolism, the methyl citric acid cycle, and the citric acid cycle during the intermediate stages of sporulation. The genes within this operon: *mmgA*, *mmgB*, *mmgC*, *mmgD*, *mmgE*, and *yqiQ* encode for proteins that have proposed biochemical roles to promote the above activities. The goal of this thesis is to biochemically confirm the activity of the protein encoded by the *yqiQ* gene. Our hypothesis is that this gene encodes for a 2-methylisocitrate lyase, the last enzyme of the methylcitric acid cycle. Methylisocitrate lyases catabolize 2-methylisocitrate into pyruvate and succinate, interconnecting the activities of the methylcitric acid cycle and the citric acid cycle of the bacterium. This pathway ultimately allows for the entrance of propionate from the metabolism of odd-chain fatty acids into the bacteria's citric acid cycle to promote ATP production.

From work done in this lab previously, *yqiQ* has been cloned, and its protein (YQIQ) was isolated and purified. In this work we optimized the yield of this purification producing approximately 2.17 milligrams per liter of culture. We are studying the "reverse" reaction by utilizing an assay which uses equimolar concentrations of pyruvate and succinate to produce 2-methylisocitrate (1). The reverse reaction is being conducted due to the lack of commercial

availability of 2-methylisocitrate.  Results have been able to provide partial evidence of this

conversion over time.  In order to investigate the proposed "forward" reaction mechanism, we

are also working on the chemical synthesis of 2-methylisocitrate.

CHARACTERIZATION OF THE BIOCHEMICAL ACTIVITY OF THE OPEN READING FRAME

*"YQIQ"* OF *BACILLUS SUBTILIS*

by

William T. Booth, II

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
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Master of Science

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2011

Approved by

_____
Committee Chair

To all those who have influenced and supported me through this process as I continue to

transcend academically, I thank you from the bottom of my heart.

APPROVAL PAGE


This thesis has been approved by the following committee of the Faculty of The

Graduate School at The University of North Carolina at Greensboro.




Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee


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Date of Final Oral Examination

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TABLE OF CONTENTS

LIST OF FIGURES

# CHAPTER I

## *BACILLUS SUBTILIS* AND THE PROCESS OF SPORULATION

### I.A  Background Information on *Bacillus Subtilis*

*Bacillus subtilis*, also known as hay bacillus, is a Gram positive, non pathogenic bacteria that is commonly found in soil, marine and freshwater sediments, certain foods, and cases of food poisoning (7). This organism is also utilized in the commercial production of antibiotics and surfactants (4). The bacterium is also one of the more dominant forms of bacteria found in seawater in polluted saltwater costal sites (7).  *B. subtilis* is commonly chosen as a model organism because it is highly accessible to genetic manipulation, and in 1997 it was the first Gram positive bacterium to have a completed genome sequence (5).  At publication, the genome was said to contain 4,214, 810 base pairs and 4100 protein coding genes (5).  This organism is considered "robust" and will grow readily in most media, and can be easily isolated for study (7).

### I.B  Explanation of the Process of Sporulation

One of *B. subtilis's* most significant characteristics is its ability to sporulate.  The bacterium has been a model organism for the study of sporulation for the past 62 years (7). The process of sporulation generally occurs during times of starvation and/or high bacterial density in which the bacterium produces a dormant, heat and chemical resistant spore (8).  When conditions return back to a state in which nutrients are plentiful, the bacterium returns back to its vegetative state (7).

1

In general terms, the morphological process of sporulation initiates at the end of the exponential (vegetative) growth phase into a state known as the "stationary phase", the phase at which there is a near halt in the reproduction of the bacterium by binary fission (2). Along with factors of nutrient deprivation and bacterial cell density, regulatory signals from the Kreb's cycle, DNA synthesis, as well as DNA damage also can play a part in the initiation of sporulation (9).

*B. subtilis* undergoes a series of morphological changes during the formation of a dormant endospore. The metamorphosis is generally described in eight stages (2):

Stage zero, describes the vegetative stage, the stage at which the bacterium is actively replicating its DNA (10).

Stage one describes the stage at which the sporulation signal originates, and the DNA is stretched across the length of the cell by synthesized chromatin (10).

At Stage two, the beginnings of what is known as the asymmetric septum, a semi-permeable membrane that develops toward one end of the cell, develops to house one of the copies of DNA transcribed at the zero stage (10). At the start of the second stage, the septum only houses approximately 30 percent of the replicated DNA, but before transition into the next stage the remaining 70 percent is assisted across the septum with the help of transport protein SPOIIIE (The abbreviaton "spo" describes genes utilized during the sporulation process, and "SPO" describes proteins) (10).

Stage three describes the process known as "engulfment" which is when the septum eventually encases the transported genome (10). Under fluorescence microscopy, the engulfment event looks like a small cell within a larger cell. This smaller cell within the larger cell

is referred to as the endospore (10). The larger portion of this cell within which the endospore is housed is referred to as the "mother" cell (10).

Stage four describes a thickening of the newly formed endospore coat surrounding the genome.  Peptidoglycan, a common component of all bacterial cell walls, forms within the coat which reinforces it into what is referred to as the "endospore cortex" (10).

In Stage five, the coat continues to thicken from protein deposits received from the mother cell known as "small, acid-soluble spore proteins" or SASP proteins. The aggregation of these proteins on the endospore cortex is what makes the spore resilient in harsh environments (10).

Stage six describes the stage of "spore maturation". This means that the spore is at the end of its developmental stage, and is ready to be released from the mother cell (10).

Stage seven describes the apoptotic activity of the mother cell, and the mature spore's eventual release into the non-nutritive environment. The spore will return back to the vegetative state (Stage zero) when the environment surrounding the spore provides enough nutritive materials for growth and replication (10).

**I.C  Molecular Genetics Overview of Transcription Factors Involved in Sporulation**

At the start of sporulation (stage zero to stage one) Krebs cycle regulatory signals along with transcription factors "sigma A and H" initiate a series of phosphate group transfers known as the phosphorelay (4,8).  The relay includes the activation of five consecutive kinases , known as kinases A through E, that allow for the passage of a phosphate group from kinase A to the integral sporulation activation protein, SPO 0A (9).  Upon phosphorylation the transition from stage one to stage two begins with the activation of transcription factors sigma-E and sigma-F.

Both factors are considered to be in an inactive state until the formation of the asymmetric septum (8).

During Stage two, Sigma-F relegates itself to the smaller portion of the asymmetrical split, while Sigma-E is produced in the mother cell in an inactive form known as "pro-sigma E"(7,8). Once Sigma F is activated it promotes the transcription of the gene spoIIR to produce the protein "SPOIIR". SPOIIR binds to and activates a septum membrane protein known as "SPOIIGA" (8). Upon activation, the removal of a 27-amino acid peptide strand from pro-sigma E converts it to sigma E. The Sigma F/E interaction plays a major role in the formation of the septum as well as providing direction for the replicated DNA to move toward its endospore, which is supplemented by SPOIIIE as stated in the previous paragraph.

As the transition from stages two to three begin, sigma E activates another sporulation transcription factor known as sigma G (8). Sigma G is found in the newly developed endospore, and only becomes active when engulfment is complete. Sigma G activates the transcription of the spoIVB gene to express for the SPOIVB protein (8). This protein binds to an endospore surface protein complex composed of proteins BofA, SpoIVFB, and SpoIVFA , and provides a means for the activation of the mother cell transcription factor, sigma K (8). Sigma-K, the last transcription factor in this cascade, has similar properties to sigma E in that sigma K converts from an inactive form "pro-sigma K" until it is activated by the surface complex (8).

This allows for transition into the latter stages of sporulation, maturation stages four through six (9). Sigma K activates what is known as the sigma K regulon, which is a series of genes involved in the formation of the spore coat as well as maturation of the spore (8).

At the conclusion of sporulation, stage 7, the process of apoptotic activation occurs, which is poorly understood (6).

## CHAPTER II

## EXPLANATION OF THE RESEARCH

### II.A  Introduction to the $\sigma^E$ Dependent, *Mmg Operon*, and Current Findings

This research project is based upon the discovery of the sigma E-dependent mother cell

metabolic gene operon (*mmg operon*) by Edward Bryan et al. (4). The objective is to gain a

clearer chemical understanding of the biochemical activity of the *mmg operon* during the

intermediate stages of sporulation. The proteins encoded by the six open reading frames (ORF's)

of this operon are similar to genes that encode for proteins involved in fatty acid metabolism

(*mmgA-C*), as well as proteins of the methyl-citric acid cycle(*mmgD,E,* and *yqiQ*) (2).

Currently, the Reddick group is working towards characterizing each of the mmg open

reading frames to biochemically define their activity. So far, ORF *mmgA* was successfully cloned,

purified, and characterized as an acetoacetyl-CoA thiolase (4). *MmgB* has been cloned and

purified, but no activity has been identified (11).  *MmgC* has been cloned and purified, and has

been shown to have acyl-CoA dehydrogenase activity (11). *MmgD* has been cloned, purified, and

characterized as a citrate and methylcitrate synthase by Rejwi Acharya (1). *MmgE* has been

cloned, purified, and is currently being characterized by Grant Hardesty. *YqiQ* has been cloned

and purified, but only partly characterized.  Figure 1 provides a pictorial representation of the

organization of the *mmg operon*.

**Figure 1.** Pictorial representation of the *mmg operon* (4)

### II.B General Biochemical Explanation of Activity of the *Mmg Operon* and Current Findings

Chemically, the *mmg operon* is proposed to have a function in the integration of activity between the citric acid cycle, methyl citric acid cycle, and the process of fatty acid metabolism, all producing elements that lead to the electron transport chain which promotes the formation of adenosine triphosphate (ATP). ATP is a necessary molecule in the progression of almost all biological processes. The proposed chemical mechanism of action of each gene within the operon is described in Figure 2. The diagram describes the proposed progression of propionate through the three cycles. It starts from its liberation from the odd chained fatty acid and its transfer into the methyl citric acid cycle. At the conclusion of the methyl citric acid cycle, *yqiQ* is projected to act as a 2-methylisocitrate lyase (2-methylisocitrate is eventually synthesized from the propionyl group) which cleaves 2-methylisocitrate into pyruate and succinate. These catabolites are components of the citric acid cycle necessary for energy production during the intermediate stages of sporulation. The net result is the oxidation of propionyl CoA to pyruvate.

So far, confirmed activity of these processes were identified in 2008 by Spencer Russell, from the Reddick lab, in which he was able to provide evidence to support that *MMGB* and *C* played a role in the metabolism of fatty acids (9). In a paper published from the same lab in 2008, Jamye Williams was also able to provide evidence that *mmgA* encodes for an enzyme that

acts as a β-ketoacyl thiolase, which likely plays a role in the formation of propionyl CoA from the metabolism of odd chained fatty acids (8).
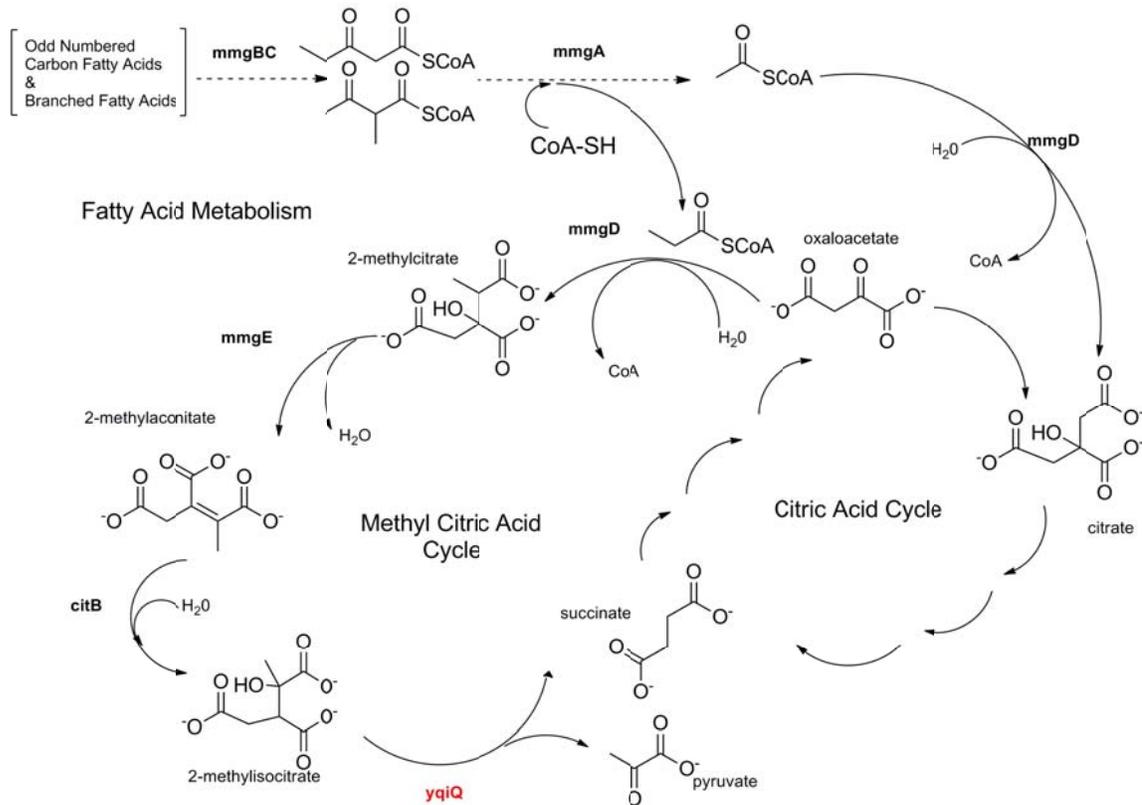


**Figure 2.** The proposed biochemical pathway encoded by the *mmg operon*

It was concluded that *MMGA* plays a role in the entrance of propionyl CoA into the bacteria's methyl citric acid cycle (8). In 2009, Rejwi Acharya, found evidence that *mmgD* encodes for a protein that performs a dual role and supports the activities of the methyl citric acid and citric acid cycles. It was shown that the enzyme facilitates the first step of the methyl citric acid cycle, the formation of methylcitrate utilizing propionyl-CoA and oxaloacetate as substrates. *MMGD*'s secondary role is in the synthesis of citrate, a component of the citric acid cycle, using acetyl -CoA and oxaloacetate as substrates.  *MMGE* is proposed to play a role in the

conversion of 2-methylcitrate into 2-methyl aconitase by an elimination of water. This gene has

recently been cloned and over-expressed in our lab.  The enzyme CITB is the only aconitase

found in *Bacillus subtilis* and is considered to play a role in the rehydration of 2-methylaconitate

to form 2-methyl isocitrate.  The research done on the enzyme *yqiQ*, the focus of this thesis, has

only been able to provide partial evidence of its catabolic activity.

In comparison to the *mmg* operon, the *prp* operons found in microorganisms like *E. coli*

and *Salmonella* species, have been found to have DNA sequence homology and are both

considered to encode for the methylcitric acid cycle(3). The *PRPB* protein also acts as a 2-

methylisocitrate lyase in *E.coli*, and has sequence homology with *yqiQ* at approximately 43

percent (2, 15). We have utilized these similarities as a guide to aid us in experimental setup.

**CHAPTER III**

**EXPERIMENTAL SECTION**

### III.A  Cloning of *yqiQ* Gene (Overview)

The original cloning and isolation of the *yqiQ* enzyme was conducted by Amy

Quattlebaum and Suzette Mills in 2008. The process was conducted by designing DNA primers

for polymerase chain reaction (PCR) amplification of *yqiQ*.  This product was ligated into a pET-

28a, a commercial plasmid, in-frame with a series of nucleotides that encoded for a long series

of histidine residues also known as a "His-tag" (This His-tag will be described in the next section

which focuses on the isolation and purification). In order to clone the gene of interest,

sequence-specific enzymes known as restriction enzymes are utilized to "cut" the desired

section of DNA from the PCR product in order to transfer the desired gene into the plasmid.  The

newly synthesized DNA and plasmid were connected together utilizing DNA ligase. Upon

ligation, the plasmid was transformed into a commercial strain of BL21(DE3) *Escherichia coli* (*E.

coli*), a genetically modified strain of *E. coli* that will not destroy foreign DNA. The *E. coli* was

placed on Luria-Bertani agar (LB agar) plates infused with Kanamycin (an antibiotic), and

incubated. Upon the growth of transformed bacterial cell colonies, a colony was removed and

placed in liquid LB/Kanamycin media for the purpose of creating a culture of the *E.coli*.   This

particular bacteria strain was used as a vehicle for increased over-expression of the *yqiQ*

protein, which will be explained in greater detail in the protein expression and isolation section.

### III.B  The pET-28a Plasmid System

The plasmid system, pET-28a (shown in Figure 3), is a vector used for the purpose of

protein expression in *E. coli*. As the diagram illustrates, the system is divided into four major

areas: the "origin of replication" (ori-3286) ensures that the plasmid is copied by the dividing *E.*

*coli*. The next major area is referred to as the "lac repressor" (lacI 773-1852) which encodes for a

repressor protein that prevents the expression of the new genetic materials and controls

transcription of the third major site known as the multi-cloning site (Bpu 1102). This site is the

location in which the *yqiq* gene was inserted.  The fourth major section of this plasmid is known

as the Kanamycin resistance gene (Kan 3995-4807).  This section aids in ensuring that only

transformed BL21-DE3 *E. coli* will grow.
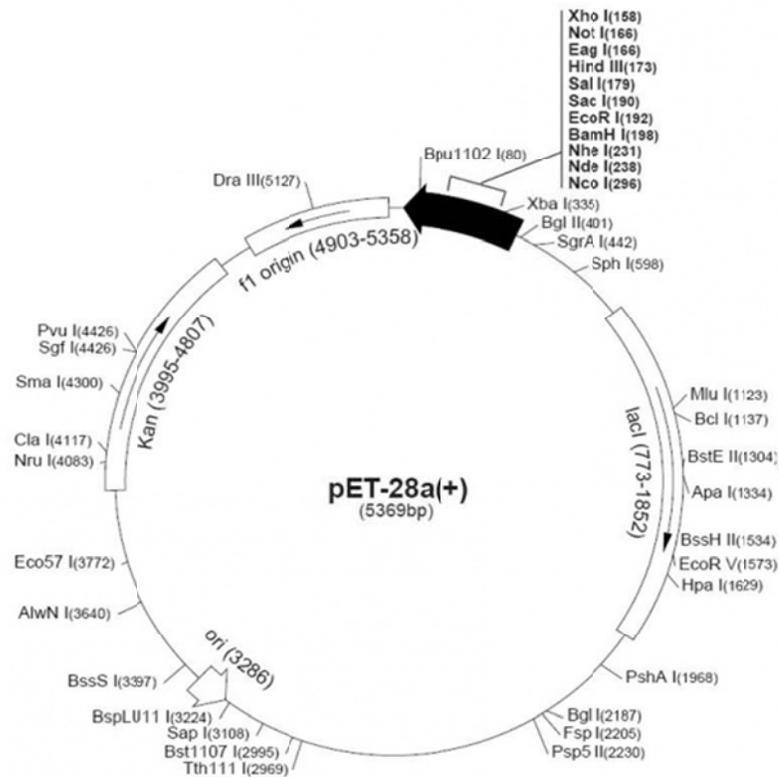


**Figure 3.** The pET-28a plasmid expression system (13)

### III.C  Culture and Over-expression of *yqiQ*

Upon growth of transformed *E. coli* on the Kanamycin agar plate, the new colonies were cultured in liquid LB media to increase the volume of *E. coli* growth. A colony is initially added to 5 ml of liquid LB media, along with an added 0.50 μl of 200 mM kanamycin, and was shaken overnight at 37° Celsius.  On the same day, a liter liquid LB media was made which was composed of 10 grams of sodium chloride, 5 g of yeast extract, 10 g of tryptone, and 1 L of nanopure water.  The pH of the solution was brought to 7.5 using 1 M solution of sodium hydroxide and continuous agitation.  After attaining the desired pH, the solution was autoclaved. Upon cooling to 25° Celsius, 1 ml of 200 mM Kanamycin is added to the solution for selection during the growth of the culture.

After overnight incubation at 18° Celsius, 2 ml from the 5 ml culture was added to the 1 L solution of LB media and allowed to incubate at 37 ° Celsius with shaking at 245 revolutions per minute (rpms).  Periodic spectrophotometeric tests to determine the rate of bacterial growth was conducted at 595 nm for approximately three hours.  Upon attaining an absorbance result of approximately 0.5, the solution was removed from the incubator to prepare for the over-expression phase.

The over-expression of the *yqiQ* gene was conducted by utilizing a synthetic analog of galactose known as Isopropyl β-D thiogalactopyranoside (IPTG). IPTG aids in the removal of the lac repressor to promote over expression of the *yqiQ* gene.  IPTG was added to the liter LB culture to give total solution concentration of approximately 1.00 mM, and was placed in an incubator, overnight, at 18° Celsius. This solution was rotated at 225 rpms and was tested the following day for the presence of the *YQIQ* protein.

### III.D  Purification of *YQIQ*

After overnight incubation of the bacterial culture preparations were made to separate

the over-expressed protein from the bacterial cell in order to isolate it. The incubated culture

was centrifuged at 7500 g at a temperature of 4 ° Celsius, for thirty minutes. After this first stage

of centrifugation the supernatant was removed, and the remaining cell pellet was prepared for

cell lysis and sonication. This was initiated by the addition of 20 ml of the binding buffer (a

solution of 0.5 M NaCl, 80 mM Tris buffer, and 5 mM imidazole at pH 8) to the pellet along with

the addition of approximately 300 mg of lysozyme. The suspension was stirred for fifteen

minutes.   The suspension was sonicated for six, thirty second periods, allowing a thirty second

rest interval between periods.  At the completion of these periods, the solution was centrifuged

for thirty minutes at 14,600 g, also at 4° Celsius. After centrifugation the supernatant was

filtered through a 0.45 μm syringe filter and loaded on a Ni-NTA nickel affinity column.

### III.E  The Nickel Affinity Column

The nickel affinity column, shown in Figure 4, is used to purify protein by utilizing a

collection of agarose beads with nickel nitrilotriacetic acid bound in a uniform fashion around

the surface of the bead.  Imidazole washes, in increasing concentrations, helps to remove bound

protein from the bead and allows for filtration through the column.   The filtered supernatant

from the cell lysis was placed in the compartment above the column and allowed to filter

through by gravity.  The passage of the supernatant through the column creates interactions

between the coordinated nickel sphere of the nickel nitrilotriacetic acid and the Histidine-tags

(His-tag®) of the over-expressed protein.  The nickel has an affinity for the nitrogens on the ring

of the histidine residues of the His-tag®, and keeps the desired protein within the column. All

non- His-Tagged proteins are more likely to filter through the column. All eluted fractions are

collected separately and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE).
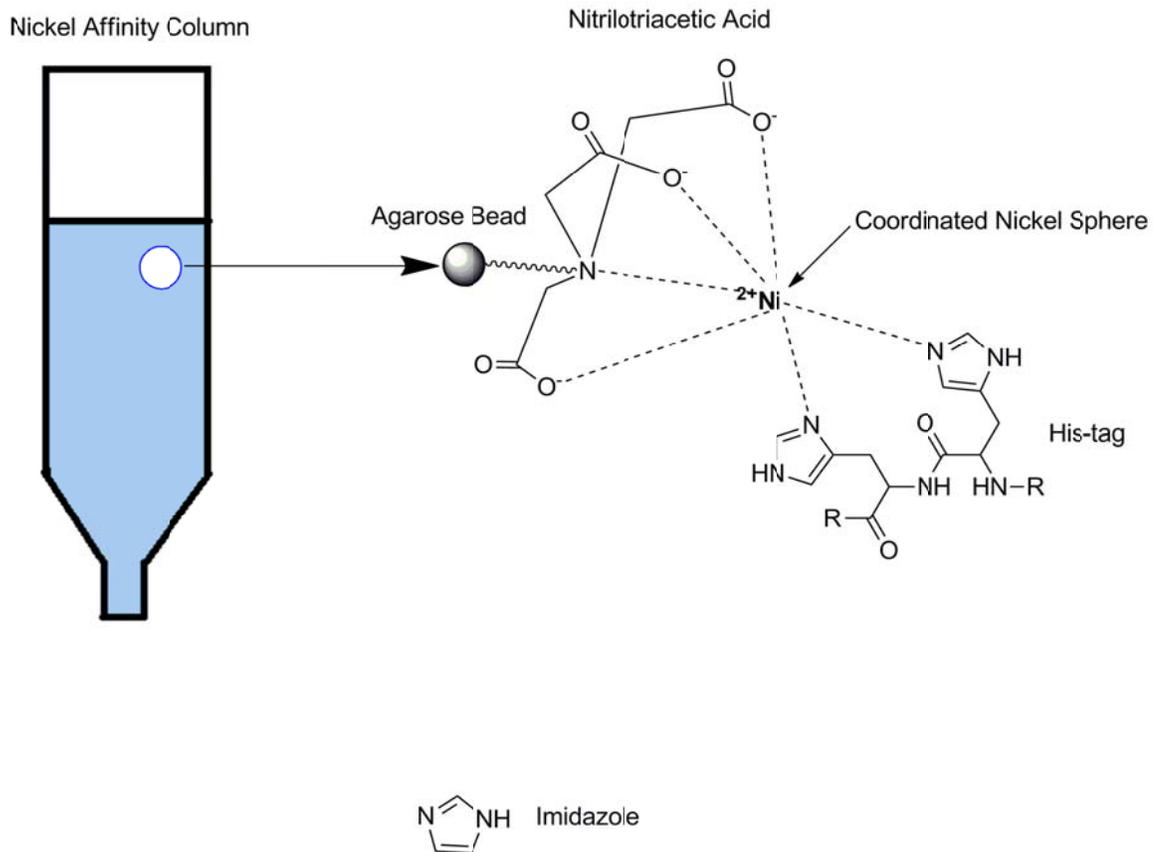


**Figure 4.** The anatomy of the Nickel affinity column and picture of the chemical structure of imidazole

Imidazole, also shown in Figure 4, has the same ring structure as the ring on the

histidine residue, and is used in a series of washes to first remove undesired proteins and

ultimately the protein of interest.  The use of imidazole promotes competition between the

nitrogens of the imidazole ring and the histidine residues of the protein for the Nickel atom.

Imidazole was allowed to filter through the nickel column in a series of washes at the following

concentrations: 5 mM described as "binding buffer", 60 mM the "wash buffer", and the final

wash at 1 M concentration, the "eluate buffer". After the eluate wash was conducted,

Bradford-Coomasie-Blue Protein Assay indicator was used to determine the presence of protein

in the eluate fractions. Upon indication for the blue color change, the eluate fraction was saved,

and allowed to dialyze overnight in a solution of 25 mM Tris buffer (pH 8.0) to remove excess

imidazole. After the dialysis bag was removed the next day, a small portion of the purified

fraction is denatured, centrifuged to remove impurities, and analyzed using SDS-PAGE to

confirm isolation of the desired protein.

### III.F  SDS-PAGE Preparation

To prepare the 12% gel, a solution of: 5.9 milliliters of nanopure water, 5 ml of 30%

acrylamide, 3.8 ml of 1.5 molar Tris buffer (pH 8.8), 0.15 ml of 10% SDS, 0.15 ml of Ammonium

persulfate, and 0.006 ml of tetramethylethylenediamine (TEMED) was made and added to the

gel apparatus. After solidification of the gel, a "stacking gel" was added on top of the 10% gel to

improve the quality of protein separation. A plastic sample-well comb was placed into the

stacking gel to create testing troughs for placement of the protein control ladder and samples of

protein from each collection from the nickel column.

The gel is placed within an electrophoresis instrument. SDS-PAGE running buffer (25

mM Tris buffer, 192 mM glycine, 0.1% SDS at pH 8.3) is added to the instrument. Next, 5.0

microliters of protein control ladder is added to the first trough, and then the remaining samples

are added consecutively in separate troughs. After the addition of the last sample, the

instrument is covered and connected to a power source set at 200 V, 300 mA, and 300 W. The

electrophoresis was allowed to run until the dye in the SDS loading buffer can show movement

across approximately three-fourths of the gel. When the desired point is reached, the gel is

removed and prepared for staining.

Staining of the gel is conducted by immersing the electrophoresed gel in Coomasie Stain

(a solution of methanol, water, Glacial acetic acid, and Coomasie Brilliant Blue) for thirty

minutes.  At the end of the thirty minute period, the gel is rinsed in distilled water, and de-

stained in a solution of water, ethanol, and glacial acetic acid for two fifteen minute periods.

After de-staining, the gel was ready for analysis.

### III.G  Determination of Enzyme Conversion of Succinate and Pyruvate to 2-Methylisocitrate

### III.G.1  Spectrophometric Determination of Pyruvate Concentration

The hypothesis for this research is that *YQlQ* protein acts as a 2-methylisocitrate lyase

that converts 2-methylisocitrate into pyruvate and succinate, as shown in Figure 5. The goal of

this study was to determine whether or not the activity of the enzyme correlated with our

hypothesis. A particular dilemma that slowed the progress of this study was the fact that 2-

methylisocitrate is not commercially available. However, if conditions are kinetically favorable,

some enzyme catalysts have the ability to operate in the reverse of their proposed reactions (1).

With that knowledge, we conducted an experiment to test the ability of the 2-methylisocitrate

lyase to convert pyruvate and succinate into 2-methylisocitrate, the reverse of the proposed

reaction.  This had precedent in a paper by Brock et al. which provided evidence to support this

activity, and produced 2-methylisocitrate in low yield (less than 10 %) (1).
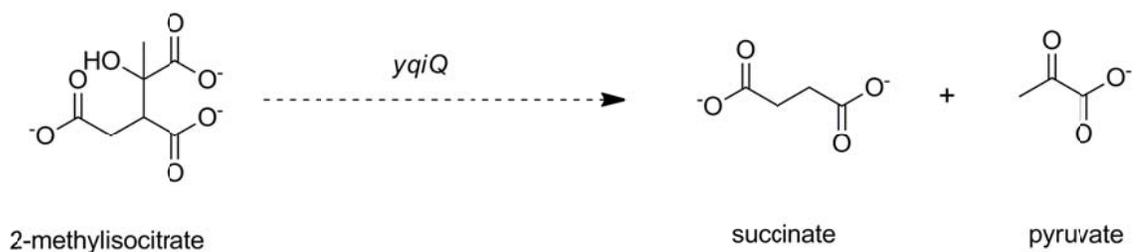


**Figure 5.** Proposed activity of *yqiQ* as a 2-methylisocitrate lyase

To determine the conversion of pyruvate and succinate into 2-methylisocitrate we utilized the reaction assay solution described in the Brock paper (2) that made use of a 10 ml mixture of 0.4 mM of Nicotinamide adenine dinucleotide (NADH), 36 U of lactic dehydrogenase, and 20 mM of HEPES buffer. The role of NADH and lactic dehydrogenase in the assay mixture was to aid in the determination of pyruvate concentration in the reaction mixture over time. Lactic dehydrogenase and NADH combined with the reaction mixture would promote a hydride transfer from NADH to pyruvate and would convert all pyruvate into lactate. NADH concentration can be determined spectrophotometrically at a wavelength of 340 nm. If the reaction mixture reacted with the assay solution and absorbance of each timed sample increased, it can be concluded that there was a decrease in the amount of available pyruvate over time. Experimentally, less pyruvate in the reaction would promote an increased absorbance, due to the fact that more NADH would be found in solution. A schematic of the assay is shown in Figure 6.
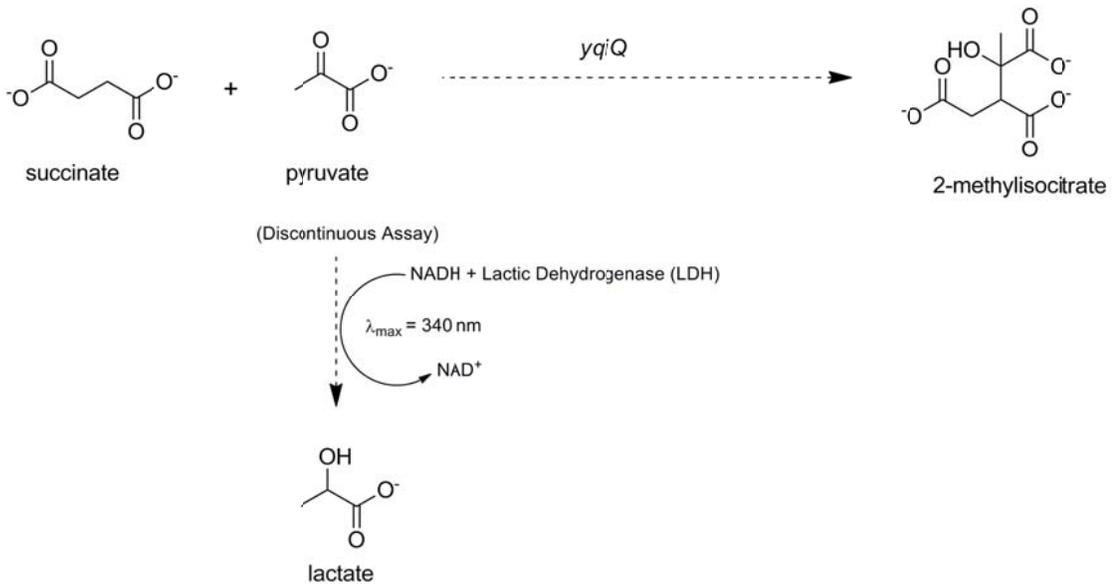


**Figure 6.** Assay for determination of pyruvate concentration

16

### III.G.2  Determination of Pyruvate Standard Curve

In order to prepare a standard curve to determine optimal activity for LDH, an assay was conducted to utilize one milliliter solutions of pyruvate, HEPES buffer, and magnesium chloride (MgCl$_2$). The concentrations of pyruvate in each 1.50 milliliter container equaled:  0 mM, 0.1 mM, 0.2 mM, 0.3mM, 0.4 mM, 0.5 mM, 0.6 mM, and 0.7 mM respectively. Four hundred microliters of pyruvate solution was mixed with 500 microliters of LDH solution (0.4 mM NADH / 36 U LDH) to give experimental solutions with pyruvate concentrations of 0.04 mM, 0.08 mM, 0.12 mM, 0.16 mM,  0.20 mM, 0.24 mM,  and 0.28 mM, respectively. Each experimental solution was measured by UV spectroscopy at 340 nanometers.

### III.G.3  *YQIQ* Reverse Reaction Assay

A  5 ml reaction mixture consisted of 400 µl of 3.75 mM pyrvuate, 400 µl of 3.75 mM succinate, 500 µl of 2.0 mM magnesium chloride, 400 µl of  1.3mM dithiothreitol, and 3300 µl of 20 mM  *4-(2*-hydroxyethyl)-1-piperazineethanesulfonic acid ("HEPES" buffer at pH 7.0 (2). At the initiation of testing, 100 µl of the *yqiQ* enzyme (0.31 mg/ml concentration) was added to the reaction mixture and was tested over a twelve minute interval, as well as daily (up to ninety-six hours).

The assay was conducted by removing a sample of the reaction mixture at timed intervals and mixing with the assay solution. This procedure was initiated directly after the introduction of *YQIQ* to the reaction mixture.  After mixing, the removal of 400 microliters of *yqiQ* reaction mixture was removed, and incubated at 90° Celsius for five minutes to denature the enzyme. Afterwards, the sample was removed and allowed to cool.  Each minute, up to twelve minutes, a sample was removed in the same fashion. All samples were conducted in triplicate. The reaction was also tested in triplicate at 24, 72, and 96 hours. Prior to

spectrophotmetric testing, the denatured samples were centrifuged to remove protein from the

solution.  The 400 µl reaction solution was mixed with 500 µl of the NADH reaction assay

solution and measured spectrophotometrically at 340 nm over a thirty second time frame. The

lowest absorbance value was recorded to reflect the actual amount of available pyruvate left in

solution.

### III.G.4  Determination of Optimum pH for Enzymatic Activity

An important goal in this experiment was to find optimum conditions for enzyme

activity. We therefore tested for enzymatic activity at increasing pH, and at increased incubation

times. The assay was designed in the same manner as stated in the previous experiment.  The

only variable that changed was the pH of the 20 mM of HEPES buffer in the reaction mixture.

The pH values tested were: 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0.

### III.G.5  Determination of Enzymatic Activity at Increased Pyruvate and Succinate Concentrations

With the knowledge gained from the previous two experiments, it was confirmed that

the concentration of pyruvate had decreased over time. It was also recognized that the process

was highly inefficient. Attempts were made to increase the concentration of available pyruvate

and succinate to 200 mM molar and 100 mM quantities, respectively in order to increase the

mass of the product formed. Conducting an assay of this type could also provide a greater

concentration of 2-methylisocitrate for confirmation by nuclear magnetic resonance

spectroscopy (NMR). The tests were conducted using the same experimental setup as described

in the first assay.  At the point of reaction mixture setup, the concentrations of pyruvate and

succinate were brought to 200 mM and 100 mM, respectively. At that point, *yqiQ* was added,

and collection was conducted in the same manner as mentioned previously, except that the

collection times were every fifteen minutes for an hour.   The maximal assay concentration of

pyruvate, according to our standard curve, was 0.12 mM pyruvate in the reaction mixture, so

after centrifugation, the sample was diluted by taking 2.5 microliters of the timed samples and

diluting them using 997.5 microliters of HEPES buffer (pH 7.5).

### III.H  Synthesis of 2-Methylisocitrate

It was anticipated that the 9 percent (0.35 mg) theoretical yield of synthesized product

would have been too small for analysis by NMR.  There was an even lower measured yield

(~0.13 percent/ 0.0053 mg) when the substrate concentration was increased.  In order to

determine the mass of synthesized product, mass spectrometry could have been used, but it

was decided to forgo spectral analysis of the reaction, and synthesize the starting substrate to

begin testing the forward reaction. The next goal was to synthesize this compound following a

procedure from Brock et al. (2001). Through the synthesis of 2-methylisocitrate, our assumption

is that upon interaction with the *yqiQ* protein we will be able to produce pyruvate and succinate

in greater amounts for confirmation by NMR. Currently, work is still being done to synthesize 2-

methylisocitrate.  The synthesis of the compound is a six step process that produces five

intermediate compounds (Figure 7): Cis-epoxymethylsuccinate disodium salt (**3a, 3b, 3c, 3d**),

Cis-epoxymethylsuccinic acid (**4a, 4b, 4c, 4d**), Dimethyl cis-epoxymethylsuccinate (**5a, 5b, 5c,

5d**), 2-methyl-5-oxo-2,3,4 tricarbomethoxytetrahydrofuran (**6a,6b, 6c, 6d**), and Erythro 2-

methylisocitrate lactone(**7a, 7b, 7c, 7d**) (1). The synthesis of the target compound would be

composed of a racemic mixture of a total of 4 stereoisomers of 2-methylisocitrate initiated from

the starting compounds citriconic acid and mesaconic acid. In the reaction mechanism described

in Figure 7, citriconic acid will lead to the formation of the cis-epoxides (S,R / R,S  enantiomeric

pairs), and mesaconic acid will lead to the formation of the trans epoxides (R,R/ S,S

enantiomeric pairs).
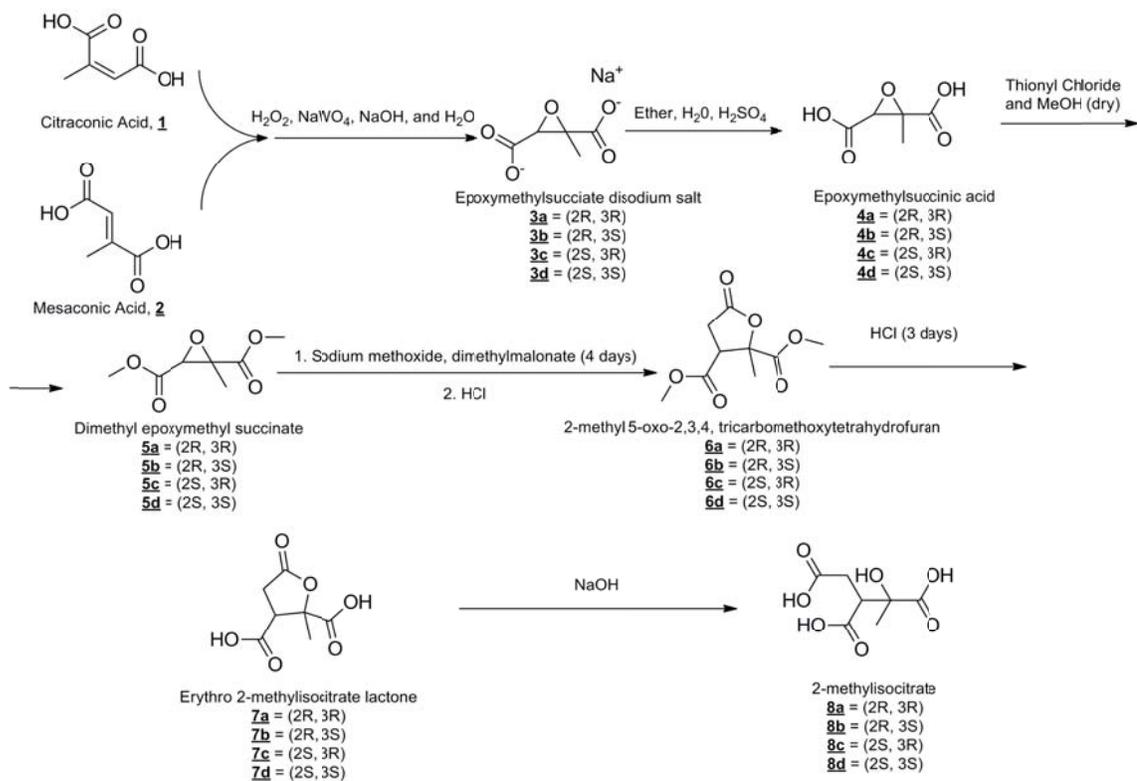


**Figure 7**. Reaction mechanism for the synthesis of the 2-methylisocitrate

### III.H.1 Synthesis of Cis-epoxymethylsuccinate Disodium Salt

The synthesis of cis-epoxymethyl succinate disodum salt (**3a-d**) was conducted utilizing a

procedure from the Brock paper. In a round bottom flask, at 0° Celsius, 15.23 ml (19.0 g/ 170

mmol) of citriconic anhydride and water (60 ml) were added and allowed to mix utilizing a

magnetic stirrer (2). Sodium hydroxide (10 g dissolved in 15 ml water) was then added while

monitoring the temperature to ensure that it did not exceed 65° Celsius (2). Upon the complete

mixture of the sodium hydroxide, dry sodium tungstate (1.70 g/ 5.0 mmol) was added to the

solution and was dissolved until completion (2). After dissolving the sodium tungstate, hydrogen

peroxide (20 ml /176 mmol) was slowly added to the solution, still monitoring the temperature

(2).  After the addition of peroxide the solution was mixed for five minutes in the ice bath to

ensure proper mixing and prevent side reactions.  After the five minute mixing, the solution was

allowed to come to room temperature (22-25 °C).  Once the solution reached room temp, the

solution was placed in a 65 °Celsius oil bath and stirred for 90 minutes (2). At the conclusion of

incubation, excess water was removed in vacuo until the solution was approximately half its

original volume (2).  At the conclusion of evaporation, the remaining solution was added to an

excess of acetone and allowed to develop a crystalline precipitate over forty-eight hours (2).

The excess acetone was filtered away and the remaining white-crystalline precipitate was

weighed at 37.52 g.  The precipitate was analyzed by NMR in solution with deuterium oxide.

**III.H.2 Synthesis of Cis-epoxymethylsuccinic Acid**

        The synthesis of cis-epoxymethy succinic acid (**4a-d**) describes only the protonation of

the carboxyl groups of the cis-epoxy methyl succinate disodium salt to produce cis-

epoxymethylsuccinic acid. Eleven grams of **3a-d** (58 mmol) was added to 20 ml of water, and

allowed to dissolve (2).  The solution was added to 300 ml of diethyl ether and stirred

continuously using a magnetic stirrer (2).  Next, concentrated sulfuric acid (6.5 ml dissolved in 70

ml diethyl ether) was added slowly to the solution and allowed to stir at room temperature for

three hours (2).  After the three hour incubation period, the solution was placed into a

separatory funnel and allowed to separate into a water layer and an ether layer (2). The water

layer was allowed to drain and was placed in a separate flask; the ether layer was drained as

well and placed in a separate flask (2).  The water layer was reintroduced to the separatory

funnel along with 100 ml of diethyl ether, mixed vigorously, and is drained and separated in the

same containers that were used previously (2).  After completion of the repeated processes, anhydrous magnesium sulfate was introduced to the combined ether layers to remove any other residual water from the solution, the solution is then filtered, and the anhydrous ether solution was collected (2).  This ether solution was placed under vacuum pressure and all excess ether in the solution is removed (2).  Upon removal of the ether, a clear oil (6.73 g) is left behind which is considered to be the desired acidic end-product. The chemical structure of the resulting oil is identified by NMR in a solution of deuterated chloroform.

### III.H.3  Synthesis of Dimethyl Cis-epoxymethylsuccinate

The synthesis of Dimethyl cis-epoxymethylsuccinate (**5a-d**) describes the methylation of the protonated carboxyl groups of **3a-d**. In order to synthesize this product 14.4 g (100 mmol) of **4a-d** was reacted with a solution of 7.50 ml (100 mmol) of distilled thionyl chloride and 100 ml of dry methanol, at 0°Celsius (2). The solution was removed from the ice bath and allowed to incubate at 25° Celsius overnight (approximately 12 hours). After the overnight incubation, the solvent was evaporated, and the remaining product was added to an excess of ether (100 ml), and was washed with 100 ml of sodium bicarbonate (1 M) in a separatory funnel (2). After mixing, the water layer was removed and the ether layer was dried using anhydrous magnesium sulfate (2).  The magnesium sulfate was filtered off leaving an anhydrous ether solution that was evaporated to prepare for flash column chromatography (2). A 3:2 hexanes to ethyl acetate solution produced an $R_f$ of approximately 0.35 and was used to run the column. The contents of collection tubes, verified by thin layer chromatography that contained the purified product were evaporated and the remaining 5a-d (11.21 g) was confirmed by NMR in a solution deuterated chloroform (2).

## IV.A  Isolation and Purification of *YQIQ*

### IV.A.1  SDS-PAGE Results

During the first purification, seven eluate collections were kept and tested against

Bradford indicator, and of the seven, two tested a positive for protein. All fractions that

provided evidence of protein were saved and placed in a dialysis bag for overnight dialysis in a

solution of 25 mM Tris Buffer (pH 8.0) for removal of imidazole. Upon removal of the bag the

next day all collected crude filtrates were analyzed by SDS-PAGE to confirm protein retrieval and

isolation of the *yqiQ* protein (results shown in Figure 8).
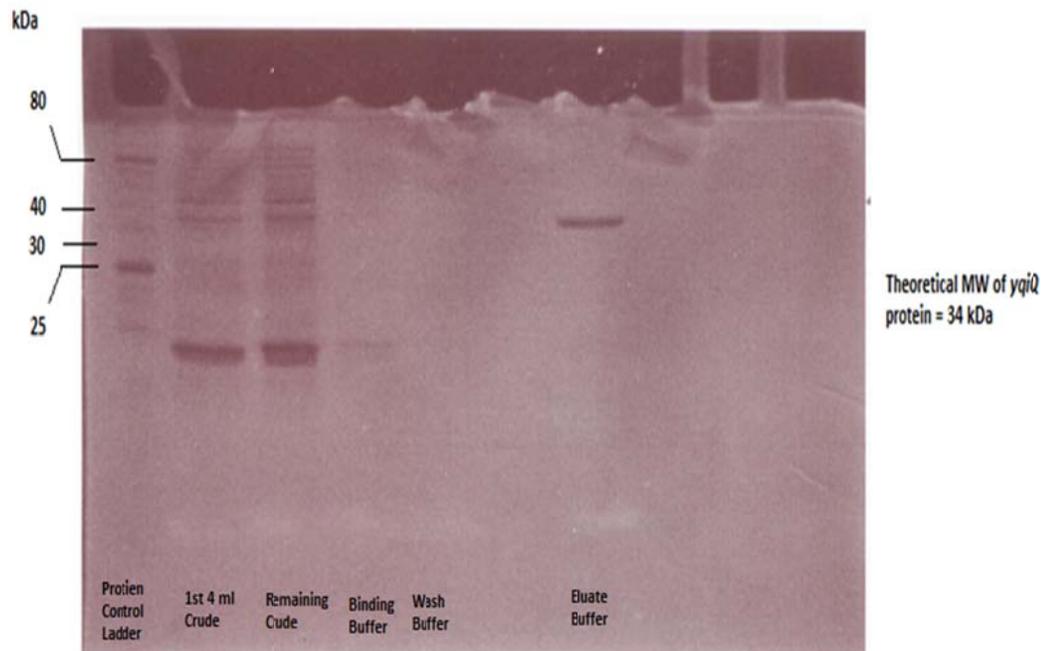


**Figure 8**. SDS-PAGE supporting the isolation of *yqiQ*

*yqiQ* has a theoretical molecular weight of 34 kiloDaltons. The SDS-PAGE showed a band

slightly above the 30 kiloDalton protein of the control ladder, which provided evidence to

support the isolation of the *yqiQ* enzyme.

**IV.A.2  Optimization of *YQIQ* Retrieval**

The procedure for the isolation of the *yqiQ* was further optimized to increase the yield

of the enzyme.  The process was improved by decreasing the amount of imidazole in the eluate

buffer down to 200 mM. This decrease prevented the rapid precipitation of the target protein

from the column, which resulted in approximately 2.17 mg/L of *yqiQ* (see Figure 9).
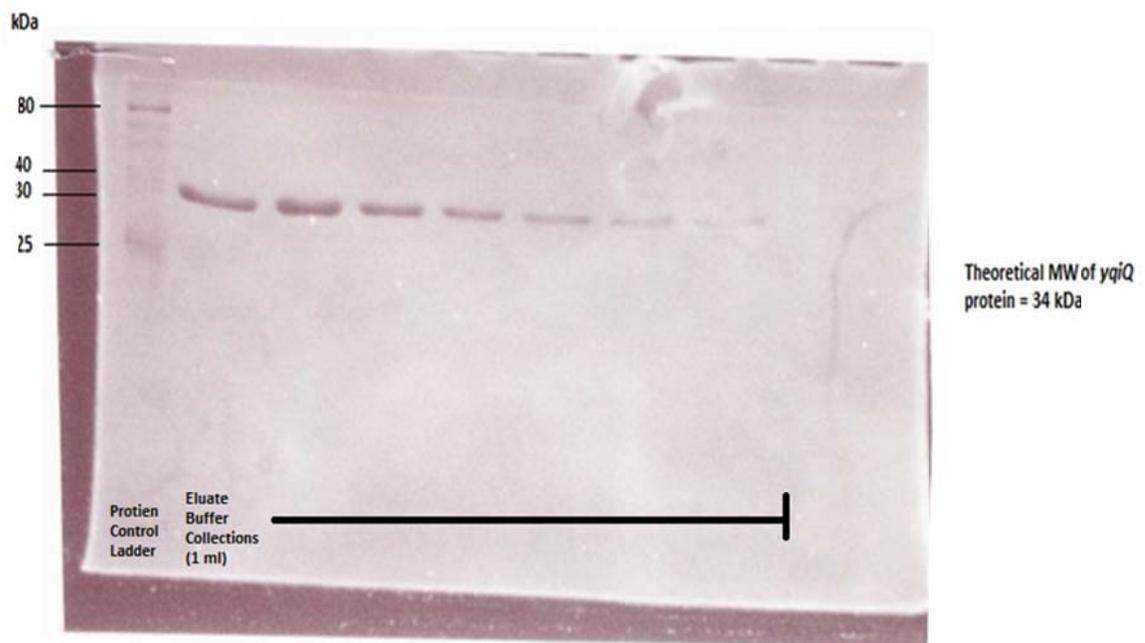


**Figure 9**. SDS-PAGE supporting increased output of pure *yqiQ* at 200 mM imidazole eluate wash
concentration

## IV.B *YQIQ* Reverse Reaction Assay Results

### IV.B.1 Lactic Dehydrogenase(LDH) Assay/Pyruvate Standard Curve

The results from the pyruvate standard curve provide evidence to support that 0.12 mM is the optimum pyruvate concentration for activity.  From this knowledge, we were able to design the reverse reaction assay that ensured the pyruvate concentration within the reaction mixture allowed for optimal LDH activity.  The pyruvate standard curve is shown in Figure 10.
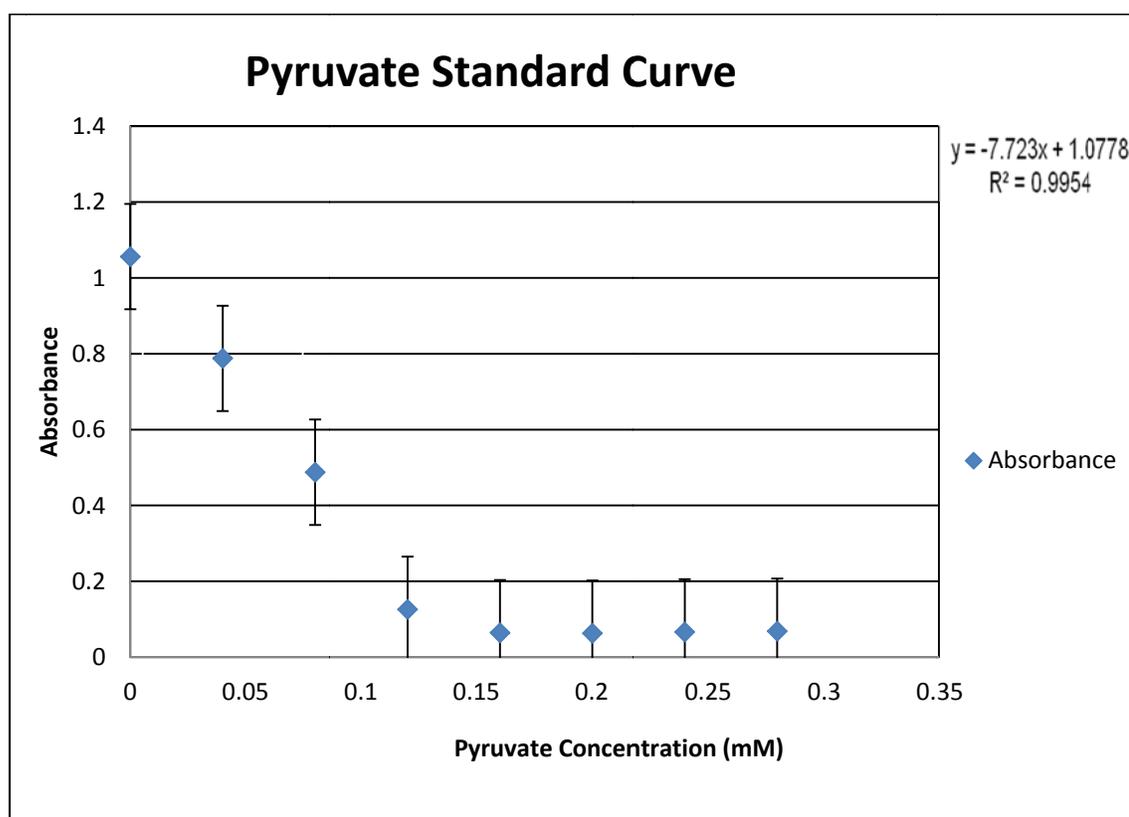


**Figure 10.** Standard curve for pyruvate

### IV.B.2 *YQIQ* Overnight Incubation Results

After some repetition and optimization, the spectrophotometric results provided evidence that the presence of the *YQIQ* enzyme promoted a decrease in available pyruvate

concentration in the reaction mixture.  Results for the 24, 72, and 96 hour incubation provided support for the decreasing amounts of pyruvate over time. Results for the twelve minute time interval provided inconsistent results and are not shown. The conclusion was that the turnover correlated well to the Brock paper, graphically determining an approximately 7% yield in desired product. This finding led us to conclude that the *YQIQ* enzyme was converting pyruvate and succinate into minute concentrations of 2-methylisocitrate.
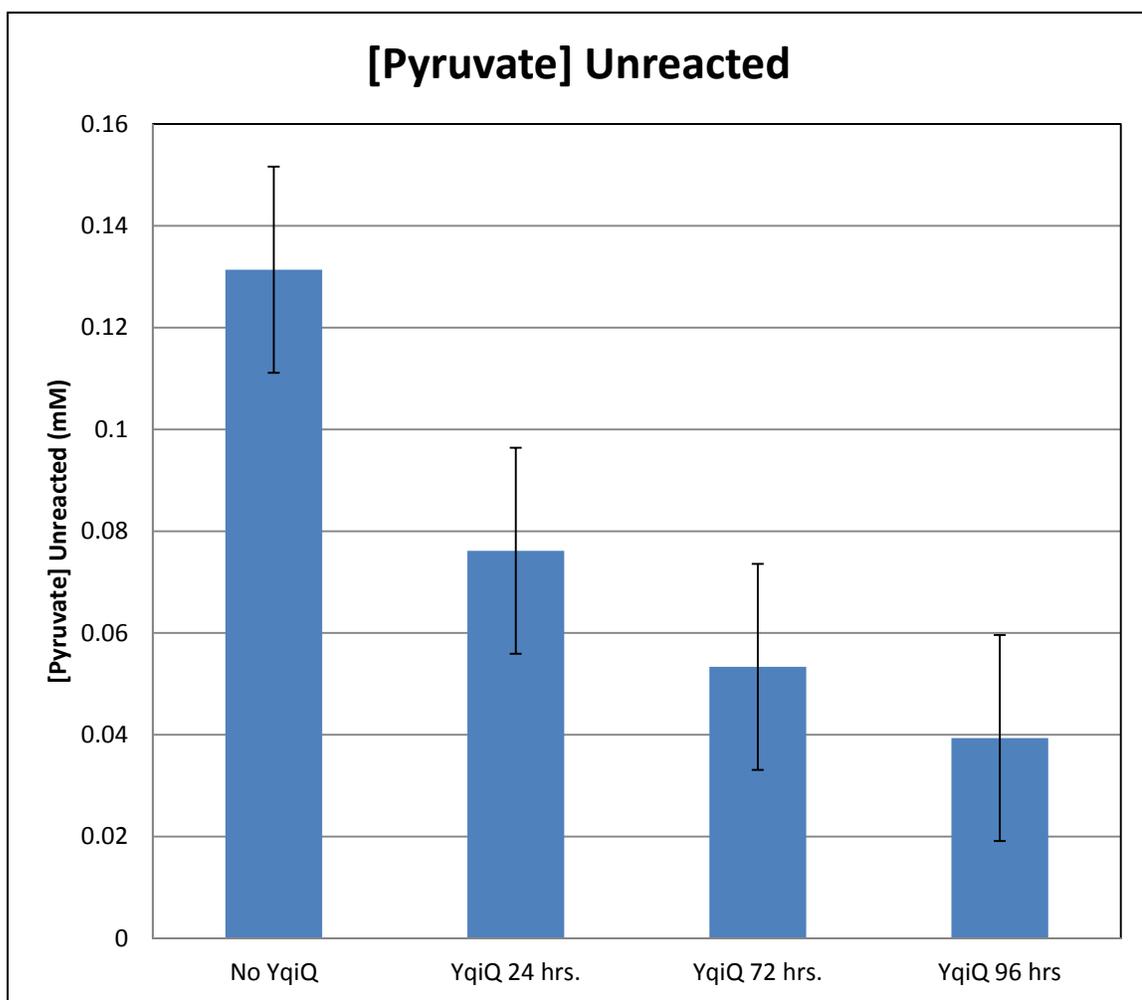


**Figure 11.** Pyruvate unreacted after 24-, 72-, and 96-hour incubation

### IV.B.3  Determination of Optimal pH for Activity of *YQIQ*

Results from this experiment allowed us to determine that the *YQIQ* enzyme had an optimal pH of 7.5 in the synthesis of 2-methylisocitrate (Figure 12). From this result, all successive experiments with the enzyme were kept at a pH of 7.5.
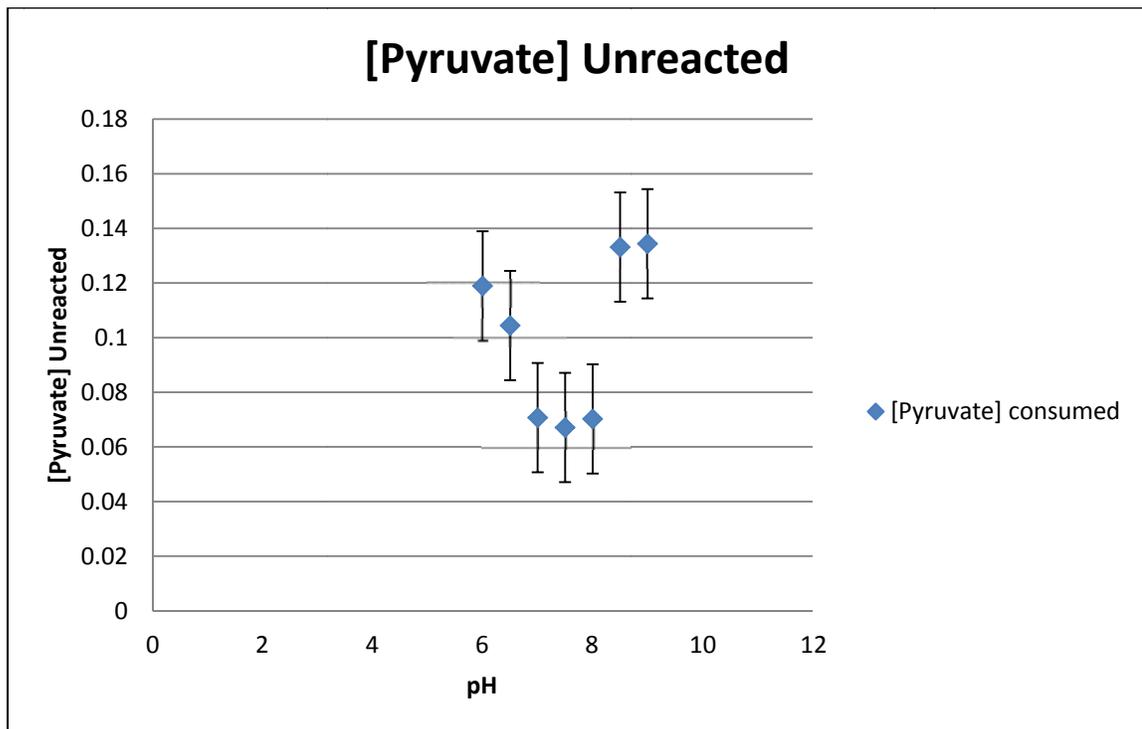


**Figure 12.** Absorbance results for pH assay

### IV.B.4  Determination of *YQIQ* Activity at Increased Substrate Concentration

As mentioned previously, the goal of this study was to increase the output of 2-methylisocitrate by increasing the reaction mixture concentrations to 200 mM pyruvate and 100 mM succinate.  The results for this assay did not correlate with the desired Michaelis-Menten kinetics, and were not as promising as the previous results.  Over an hour period, absorbance values averaged at slightly under 0.0100 to signifying that the amount of available pyruvate was

27

still high in solution, and the conversion into 2-methylisocitrate had not occurred. It was concluded that in this experiment enzymatic activity provided no measureable effect as a result of increased substrate concentration.  Results are shown in Figure 13.
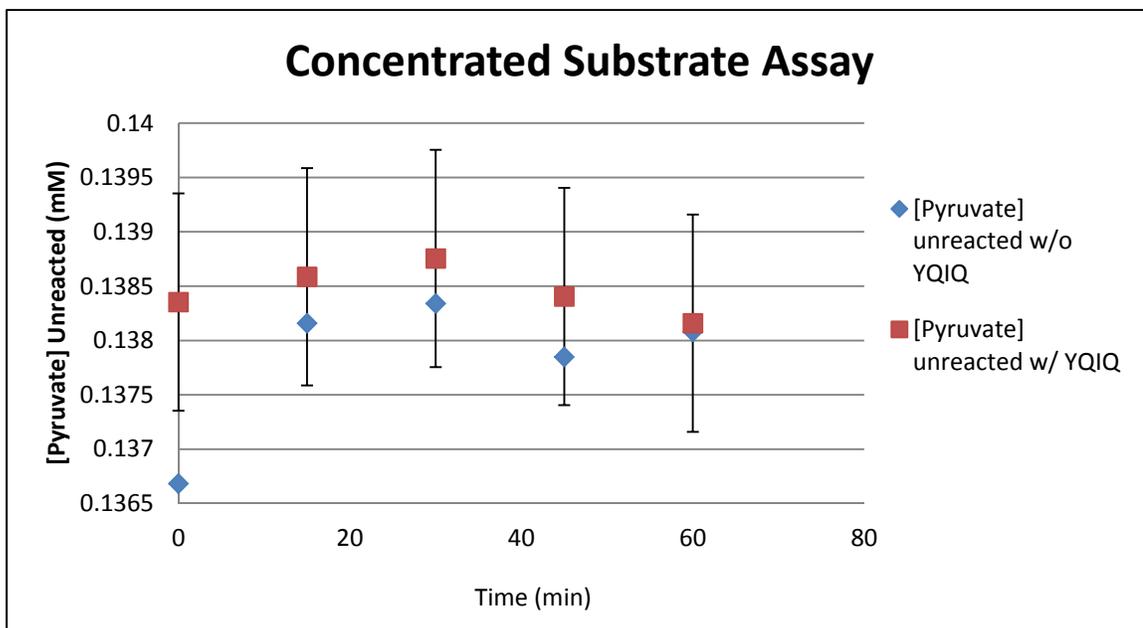


**Figure 13.** Assay results at 200 mM pyruvate and 100 mM succinate

### IV.C Synthesis of 2-Methylisocitrate Results

### IV.C.1 Results for Synthesis of Cis-epoxymethylsuccinate Disodium Salt

One dimensional proton nuclear magnetic resonance results provide evidence to support the presence of the disodium salt with chemical shift peaks as follows: ^{1}H-NMR (500Mhz, $D_2O$): $\delta$ = 1.38 ( 3H, s, $CH_3$),  3.24 ( 1H, s, OCH); ^{13}C-NMR (125 MHz) : $\delta$ = 19.57, 61.93, 63.62, 174.00, 175.47 ppms. Other major peaks in these spectra describe impurities from acetone and water from the experimental work-up.  Spectra are shown in Figures 14 and 15 and correlate with previously reported data.
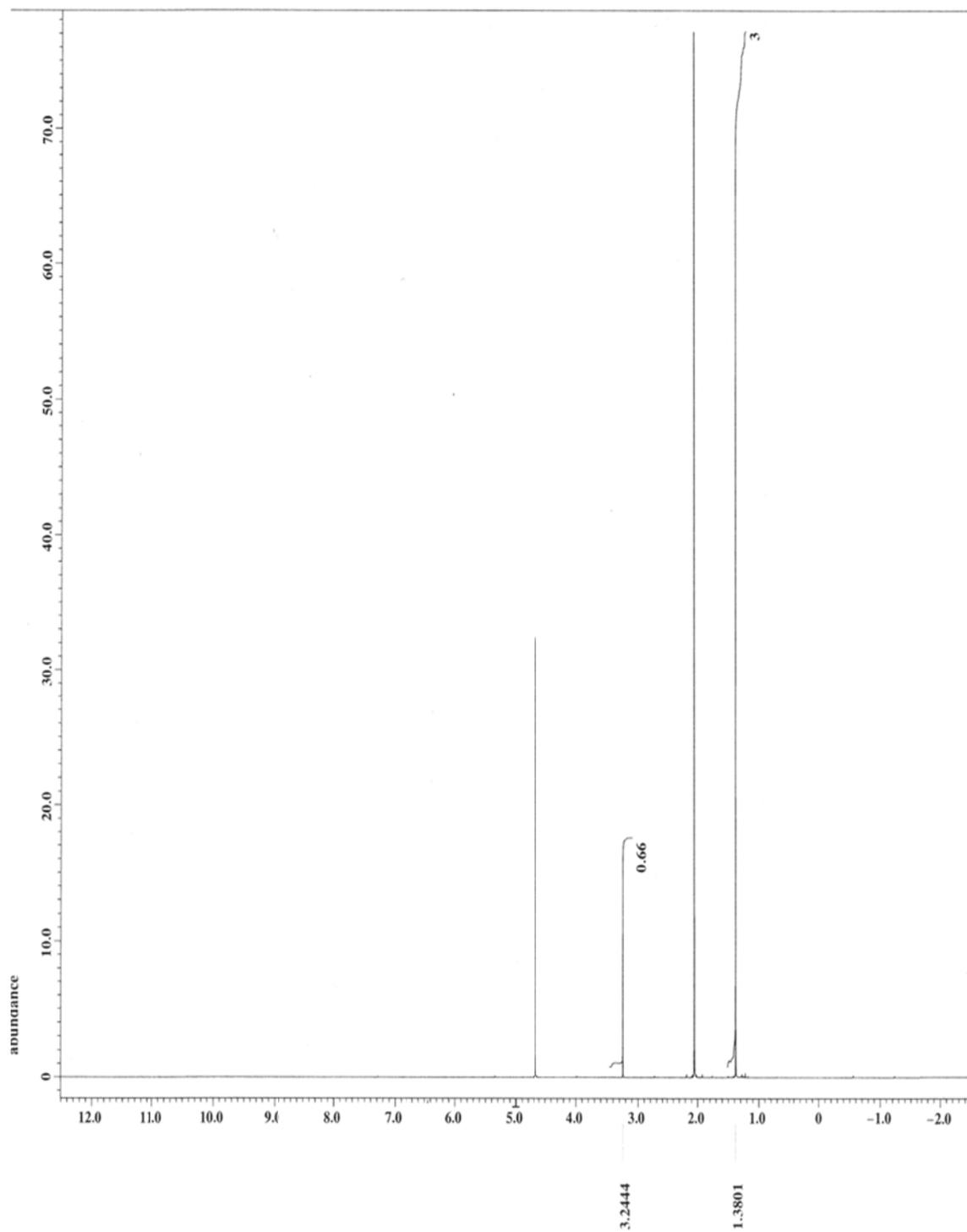
**Figure 14.** 1-D proton spectrum of cis-epoxymethylsuccinate disodium salt
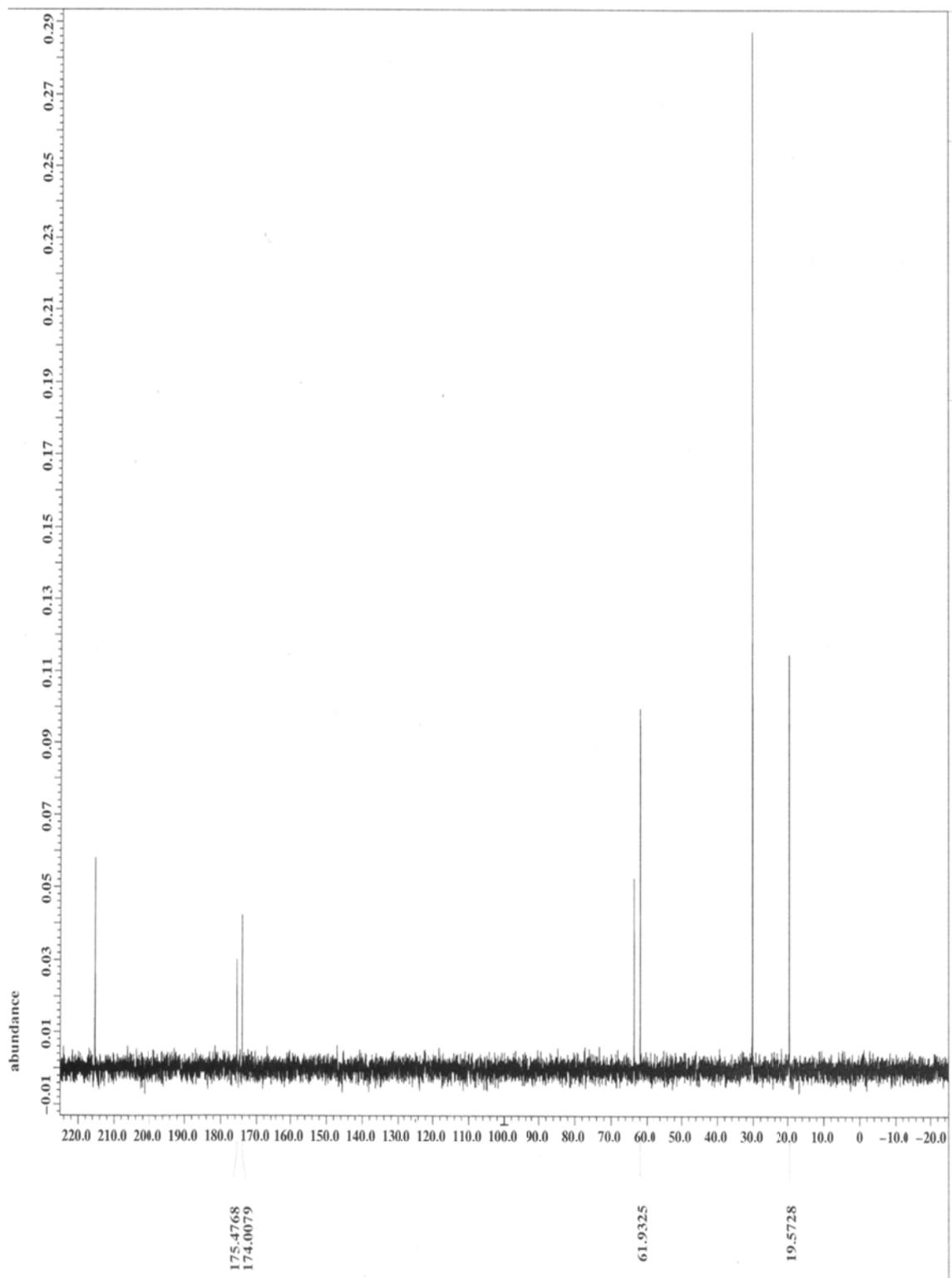
**Figure 15.** 1-D Carbon NMR of cis-epoxymethylsuccinate disodium salt

**IV.C.2 Results for Synthesis of Cis-epoxymethylsuccinic Acid**

One-dimensional proton NMR results provides evidence to support the presence of cis-epoxymethylsuccinic acid with results as follows: ^{1}H-NMR (500 MHz) $\delta$ = 1.56 (3H, s, $CH_3$), 3.57 (1H, s, OCH); ^{13}C-NMR (125 MHz, $CDCl_3$) $\delta$ = 18.40, 58.30, 60.22, 167.26, 168.52. Other major peaks in these spectra describe impurities from acetone and ether from the experimental work-up. Spectra are shown in Figures 16 and 17.
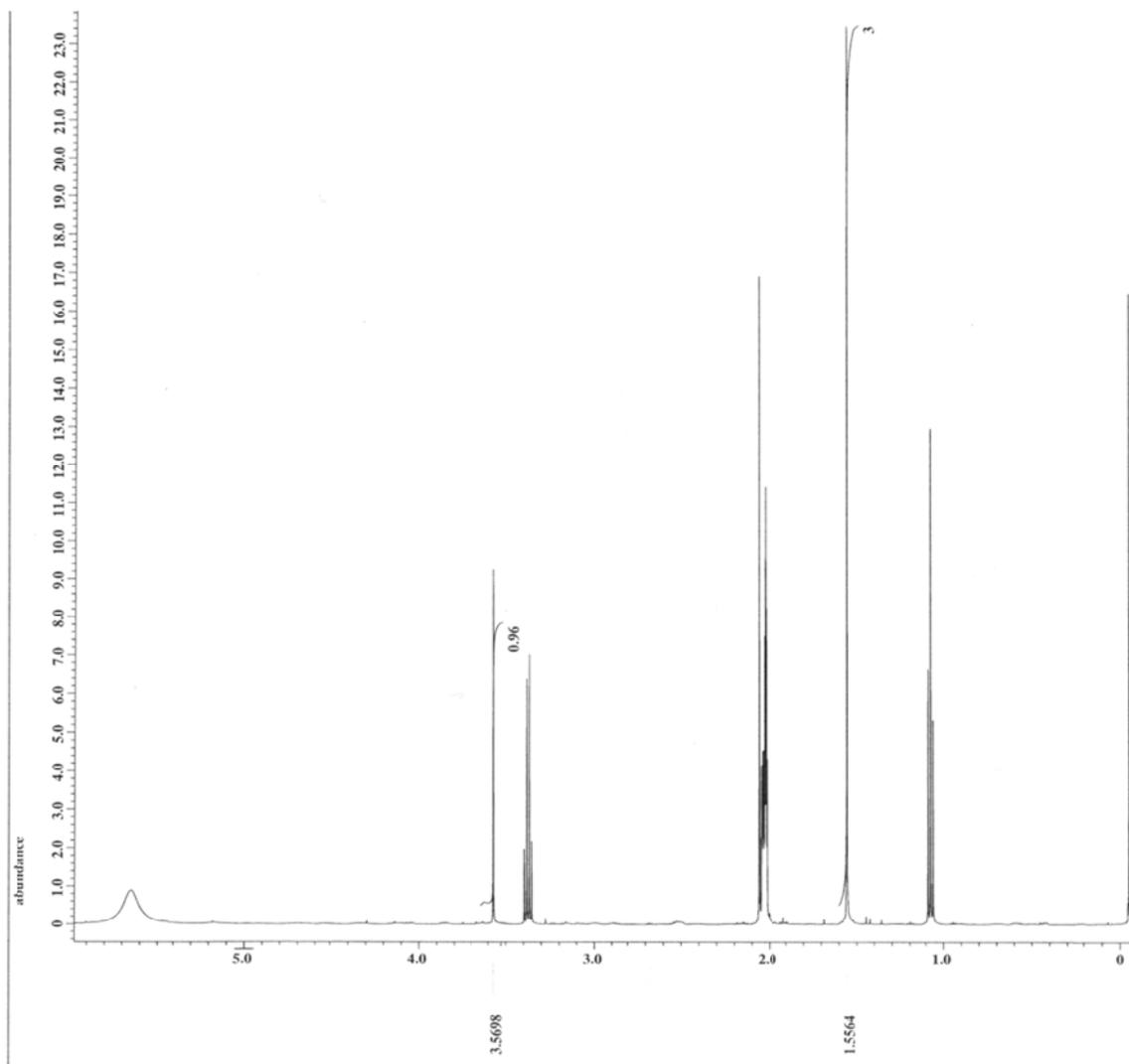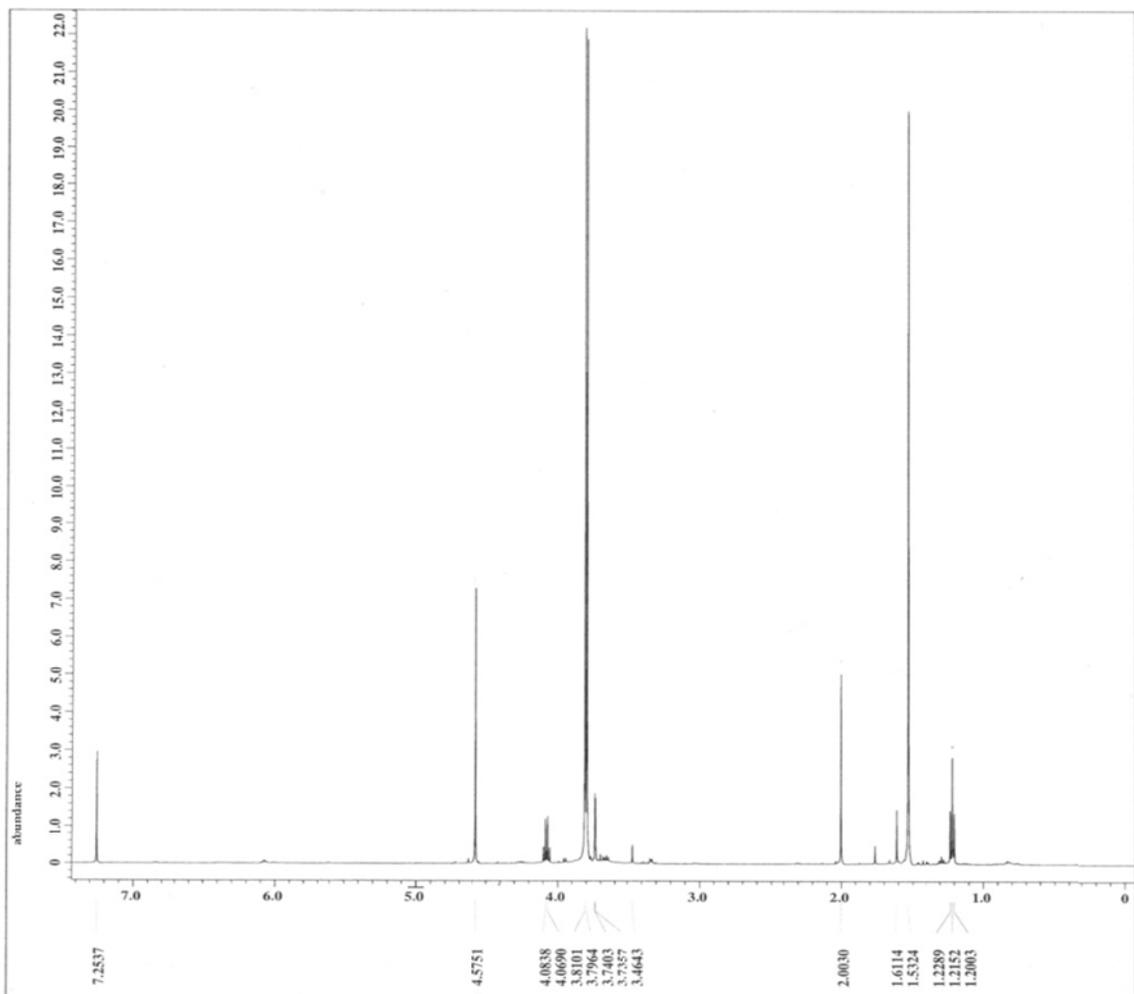


**Figure 16.** Proton NMR results supporting the synthesis of cis-epoxymethylsuccinic acid
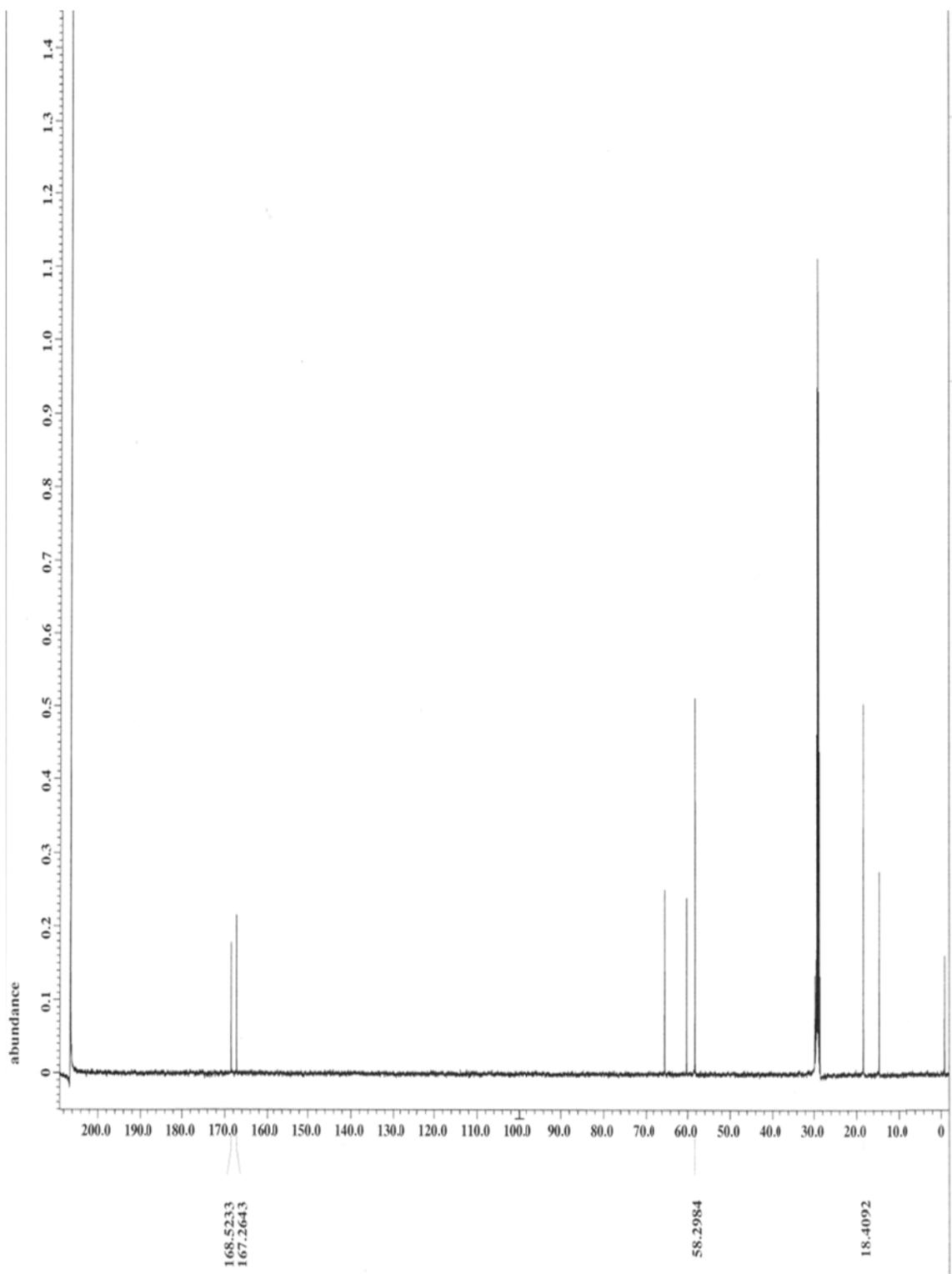
**Figure 17.** Carbon NMR results supporting the synthesis of cis-epoxymethylsuccinic acid

## IV.C.3  Results for Synthesis of Dimethyl Cis-epoxymethylsuccinate

One- dimensional proton NMR results provide evidence to support the synthesis of di-methyl cis-epoxymethylsuccinate with results as follows: $^{1}$H-NMR (500 MHz) $\delta$ = 1.53 (3H, s, CH$_3$), 3.80 (3H, s, CH$_3$), 3.81(3H, s, CH$_3$) 4.58 (1H, s, OCH); $^{13}$C-NMR (125 MHz, CDCl$_3$) $\delta$ = 23.23, 52.80, 53.39, 53.61, 61.81, 167.17, 173.29.  Other major peaks in these spectra describe impurities from ethyl acetate, hexanes, and chloroform from the experimental work-up. Proton and carbon NMR results are pictured in Figures 18 and 19.



**Figure 18**. Proton NMR results supporting the synthesis of dimethyl cis-epoxymethylsuccinate
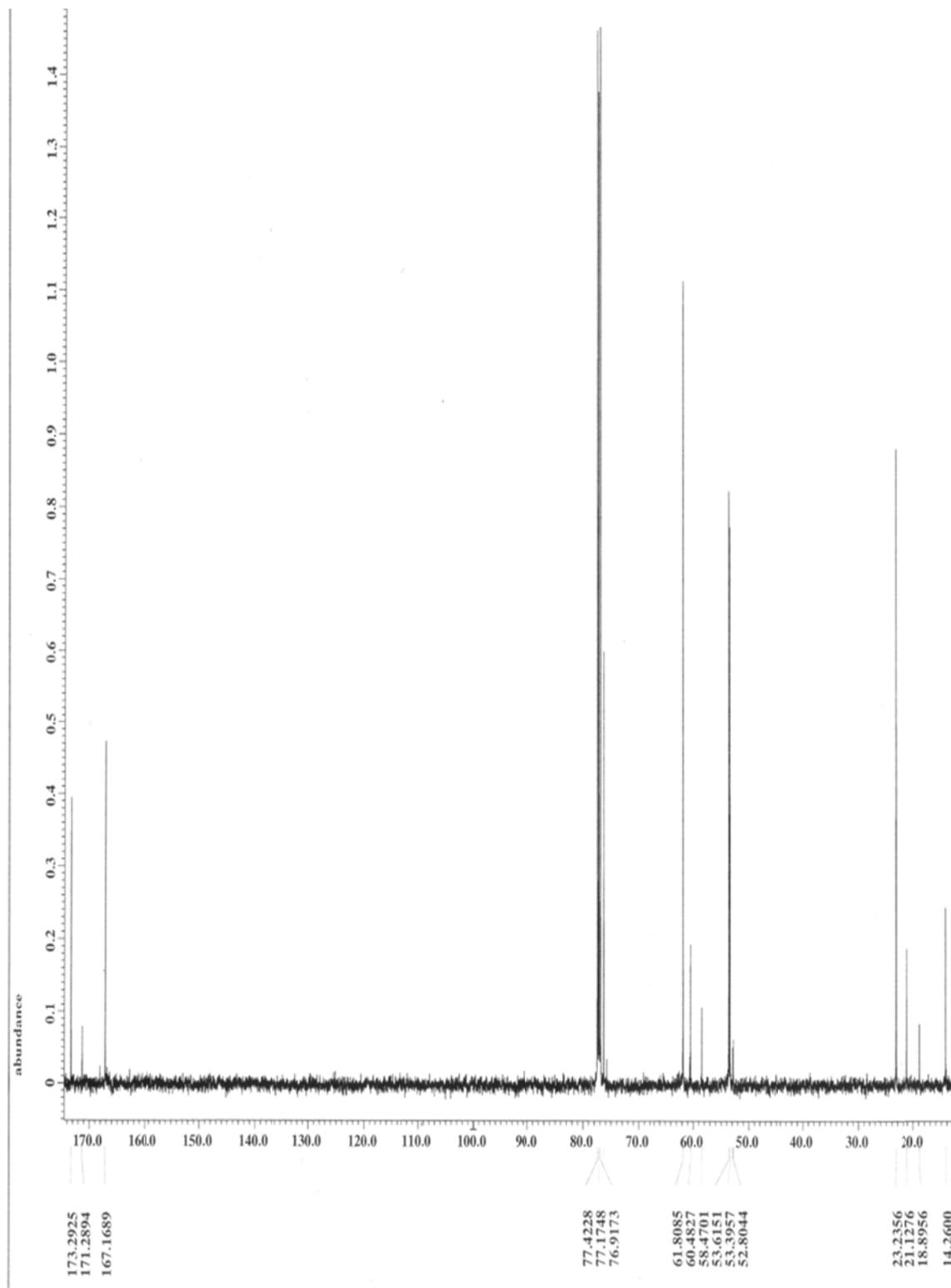
**Figure 19**. Carbon NMR results supporting the synthesis of dimethyl cis-epoxymethylsuccinate

**CHAPTER V**

**CONCLUSION**

So far, we have been able to provide partial evidence to support the proposed reaction mechanism of *yqiQ*, 2-methyl isocitrate lyase. Results from this research support that in a solution of pyruvate and succinate, the presence of *YQIQ* promotes a decrease in the concentration of pyruvate over time. This provides an increase in absorbance spectrophotometrically, due to the fact that lactic dehydrogenase has a lesser concentration of pyruvate to convert into lactate.

Successful synthesis of the first three intermediates of five to eventually produce 2-methylisocitrate has been conducted. The future goal of this project is to eventually complete the synthesis of 2-methylsiocitrate in order to test the compound in solution with *YQIQ* to attempt to confirm the forward reaction. Kinetic studies will follow to characterize the rate of conversion of the enzyme.

**BIBLIOGRAPHY**


1.      Acharya, Rejwi and Jason J. Reddick. Overexpression, purification, and characterization of MmgD from Bacillus subtilis strain 168. Thesis. University of North Carolina at Greensboro, 2009.

2.      Brock, Matthias, Dan Darley, Susanne Textor, and Wolfgang Bucknel. "2-methyisocitrate lyases from the bacterium Escherichia coli, and the filamentous fungus Aspergillus nidulans (Characterization and comparison of both enzymes)." *European Journal of Biochemistry* 268 (2001): 3577-3586. Print.

3.      Brock, Matthias, Claudia Maerker, Alexandra Schutz, Uwe Volker, and Wolfgang Buckel. "Oxidation of Propionate to Pyruvate in Escherichia Coli. Involvement of Methylcitrate Dehydratase and Aconitase." *European Journal of Biochemistry* 269.24 (2002): 6184-194. Print.

4.      Bryan, Edward M., Bernard W. Beall, and Charles P. Moran. "A Sigma E-Dependent Operon Subject to Catabolite Repression during Sporulation in Bacillus Subtilis." *Journal of Bacteriology* 178.16 (1996): 4778-786. Web.


5.      Errington, Jeff. "Regulation of Endospore Formation in Bacillus Subtilis." *Nature Reviews Microbiology* 1.2 (2003): 117-26. Print.

6.       Harwood, Colin R. "Chapter 8." *Bacillus*. New York: Plenum, 1989. 169-215. Print.

7.      Hilbert, D. W., and P. J. Piggot. "Compartmentalization of Gene Expression during Bacillus Subtilis Spore Formation." *Microbiology and Molecular Biology Reviews* 68.2 (2004): 234-62. Print.

8.       Ireton, Keith, Shengfang Jin, Alan D. Grossman, and Abraham Sonenshein. "Krebs Cycle Function Is Required for Activation of the Spo0A Transcription Factor in Bacillus Subtilis." *Proceedings of the National Academy of Sciences of the United States of America* 92.7 (1995): 2845-849. Print.

9.      Meyer, Pablo, Jennifer Gutierrez, Kit Pogliano, and Jonathan Dworkin. "Cell Wall Synthesis Is Necessary for Membrane Dynamics during Sporulation of Bacillus Subtilis." *Molecular Microbiology* 76.4 (2010): 956-70. Print.

10.      Reddick, Jason J., and Jayme K. Williams. "The MmgA Gene from Bacillus Subtilis Encodes a Degradative Acetoacetyl-CoA Thiolase." *Biotechnology Letters* 30.6 (2008): 1045-050. Print.

11.      Russell, Spencer A., and Jason J. Reddick. *Overexpression, Purification, and Characterization of MmgB and MmgC from Bacillus Subtilis Strain 168.* Thesis. University of North Carolina at Greensboro, 2008. Print.

12.      Novagen. *His-Bind Kit: Technical Manual*. TB054 ed. Darmstadt, DE: Merck KGaA, 2006. Print. Rev. F0106.

13.      Novagen, pET System Manual. 11th Edition.

14.      Schmalisch, Matthias, Elisa Maiques, Lachezar Nikolov, Amy Camp, Bastien Chevreux, Andrea Muffler, Sabrina Rodriguez, John Perkins, and Richard Losick. "Small Genes under Sporulation Control in the Bacillus Subtilis Genome." *Journal of Bacteriology* 192.20 (2010): 5402-412. Web.

15.      Sonenshein, A. L., James A. Hoch, and Richard Losick. *Bacillus Subtilis and Its Closest Relatives*. Washington, D.C.: ASM, 2002. Print.

16.      Stragier, Patrick, and Richard Losick. "Molecular Genetics Of Sporulation In Bacillus Subtilis." *Annual Review of Genetics* 30.1 (1996): 297-341. Print.

17.      Winans, Stephen Carlyle., and Bonnie L. Bassler. "Chapter 1." *Chemical Communication among Bacteria*. Washington, DC: ASM, 2008. 3-12. Print.