GLUTAMATE CATABOLISM AND SUCCINATE PRODUCTION IN *Fusobacterium varium*

by

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ABSTRACT

Important bacteria located in the human gut contribute to human health and disease. Further understanding of metabolism in the gut bacterium *F. varium* was developed in this thesis. The use of [$^{13}C_5$]glutamate and the analysis of ^{13}C-^{13}C coupling patterns observed by NMR spectroscopy established that glutamate was catabolized by two different pathways. The methylaspartate pathway was utilized when cobalt ions were available while the hydroxyglutarate pathway functioned when cobalt ions were not available illustrating environmental control over pathway selection. Succinate, a metabolic product of *F. varium*, was obtained in increased yield when cultures were supplemented with fumarate. Factors affecting succinate production were examined using cell suspensions. Suspensions of cells grown on fumarate-supplemented medium catalyzed high conversions of fumarate to succinate when supplied with sufficient carbon source. Glucose, sorbitol and glycerol were suitable carbon sources and efficient conversions were obtained at fumarate concentrations up to 300 mM. At high fumarate concentrations, retention of succinate inside the cells noticeably reduced the levels in suspensions fluids. This approach has potential for the production of biosuccinate acid, a substrate used for the sustainable manufacture of commodity chemicals.

LIST OF ABBREVIATIONS USED

^{1}H	Proton
^{13}C	Carbon 13
^{2}H	Deuterium
ADP	Adeosine Diphosphate
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
CoA	Coenzyme A
d	Doublet
dd	Doublet of doublets
EMP	Embden Meyerhof Parnas
ESI-MS	Electrospray Ionization Mass Spectrometry
g	Gravitational Force
GABA	γ-Aminobutyric acid
GC	Gas Chromatography
h	hour
Hz	Hertz
J	Coupling constant
MHz	Megahertz
mL	Milliliter
mM	Millimolar
MS	Mass Spectrometry

m/z	Mass to charge ratio
NMR	Nuclear Magnetic Resonance
na	Not applicable
nd	Not detected
NAD	Nicotinamide adenine dinuleotide
OD_{660}	Optical density at 660 nanometers
PBS	Phosphate Buffered Saline
ppm	Parts per million
s	Singlet
t	Triplet
TLC	Thin Layer Chromatography
U-	Uniformly labeled
v/v/v	Relative amount based on volume

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CHAPTER 1: INTRODUCTION

1.1 Gut Bacteria and *F. varium:*

The microbial community in the human gut is one of the most complex ecosystems on earth, consisting of approximately 1000 different species of bacteria.[1] The benefits of bacteria to the human body have been documented in detail and include stimulating the immune system,[2,3] protecting the body against colonization of infectious agents,[4,5] and contributing to nutrition.[6,7] Disadvantages provided by the microbial community in the human gut are associated with the accumulation of metabolic products such as indoles, phenols, and amines, which in large doses are toxic.[8,9] Overall, the benefits to the host far outweigh the negatives and due to this, the controlled manipulation of the metabolic activities of gut microbiota has drawn interest.[10,11] However, in order to manipulate the metabolic activities of gut microbia, their metabolic activities must first be understood.

One bacterial genus found within the human bowel is *Fuscobacterium*. The fuscobacteria are nonspore-forming obligately anaerobic gram-negative rods that belong to the family *Bacteroidaceae*.[12] The genus consists of both commensal and pathogenic species that fill various ecological niches found in the oral cavity, urogenital, and gastrointestinal tracts.[13,14] Despite the regular isolation of *Fusobacterium* species from diseased and healthy sites, there is limited information on their basic metabolic processes.[15,16] This is caused by the specific growth requirements and lack of adequate tools for genetic manipulation.[17,18] From information gathered, *Fusobacterium* species are capable of deriving energy by fermentation of amino acids[19,20,21] and carbohydrates.[22,23]

Fusobacterium varium is an important constituent of healthy intestinal flora and while usually nonpathogenic,[24] has been implicated in ulcerative colitis,[25] colon cancer,[25] intraocular infections, [26] and conjunctivitis.[27,28] Another species in the genus, *Fusobacterium nucleatum,* is more studied and is known to play a vital role in the grouping of infective bacteria on oral tissues in peridontal disease.[29,30] While *F. varium* is associated with stable periodontal conditions,[31] its primary habitat is the large intestine due to the availability of a rich amount of protein and other substances necessary for growth.[6,32] Metabolic products produced by *F. varium* are utilized by other members of the complex gut ecosystem.[33,34] The symbiotic microorganisms provide benefits and the indigenous flora protect the host from infection and strengthen the immune system.[4,35] *F. varium* has been shown to be protective against shigella and salmonella strains alone or in combination with other microbes.[36,37] Also, the metabolic products of various microbiota, such as *F. varium,* have been shown to play a causative or therapeutic effect in the role of various pathologies.[9,38,39]

1.2 Metabolic Products of *F. varium:*

Aliphatic carboxylic acids are major characteristic constituents in the metabolic footprints (or exometabolomes) of anaerobes.[40,41,42] Acetic and butyric acids in the exometabolome are derived from both carbohydrates and amino acids,[22,43] while degradation of specific amino acids (e.g., threonine, leucine and valine) leads to the accumulation of propionic and branched-chain carboxylic acids.[44,45] Short-chain aliphatic carboxylic acids, such as acetic acid, propionic acid, and butyric acid, are associated with beneficial colonic health effects, including disease prevention.[46,47,48]

Groups of metabolites with similar properties (e.g., carboxylic acids) are determined readily using chromatographic and electrophoretic methods.[49] Gas chromatography (GC) has been used for metabolic footprinting of agar and liquid cultures of anaerobic bacteria.[50,51] Extraction of carboxylic acids from acidified medium and headspace analysis gives equivalent results.[52] Derivatization (e.g., esterification) has proven effective for analytical techniques such as liquid chromatography and nuclear magnetic resonance (NMR) spectroscopy.[49]

F. varium produces six carboxylate products after growth in defined medium. Acetate, butyrate, formate, lactate, propionate and succinate were identified using ^{1}H NMR spectroscopy.[42] Optimization of the composition of the growth medium to support abundant growth of *F. varium* was guided by ^{1}H NMR analysis of the supernatant. In the optimized medium, group 1 amino acids (alanine, arginine, glycine, isoleucine, methionine, proline, and valine) were present at 0.2 mM, and group 2 amino acids (phenylalanine, tryptophan, and tyrosine) had concentrations of 1.5 mM. The group 3 amino acids (asparagine, aspartate, cysteine, glutamate, glutamine, histidine, lysine, serine, and threonine) were supplied at the higher concentration of 2.0 mM.

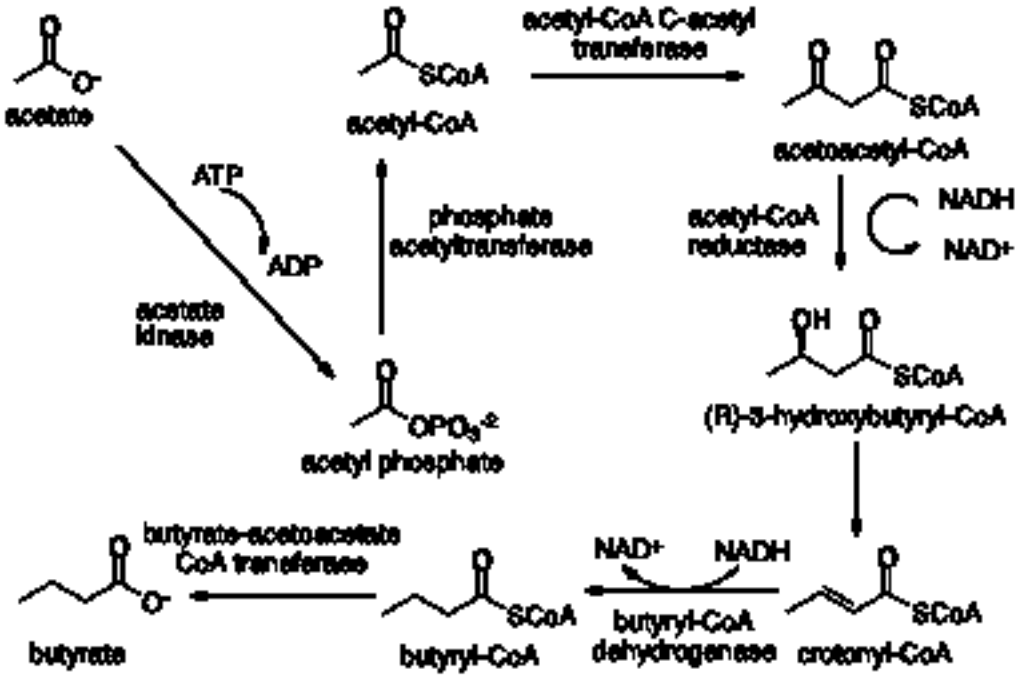
In other experiments, it was found that the carboxylate end products were also derived from glucose.[42] The oxidative catabolism of glucose under anaerobic conditions yields NADH. In fusobacteria, reductive processes form lactate and possibly succinate, and the conversion of acetate into butyrate yields NAD^{+} as a co-product. Therefore, when energy is derived from glucose, there is production of butyrate and lactate.[40]

The previous study[42] showed that the amino acids required in small amounts, such as group 1 and 2, are probably used as substrates for essential metabolism, whereas the

amino acids required in large amounts, such as group 3 are utilized as energy sources.[40] Since acetate and butyrate products were found in all culture conditions, it can be surmised that the acetate-butyrate conversion pathway provides a central role. The results also show that *F. varium* is versatile, being able to produce acetate and butyrate in a variety of different conditions.[40]

Acetate is the most populous short chain fatty acid found in the gut and is very important for maintenance of colonic health as well as acting an energy source for colonocytes. Butyrate is also a short chain fatty acid that is utilized by colonocytes.[53] It exerts anti-inflammatory effects and has been implicated in protection against inflammatory bowel disease and cancer.[38,54] With more understanding of the role of butyrate, it could be beneficial for colon health.

The formation of acetate and butyrate in anaerobic bacteria is associated with energy production and coenzyme regeneration pathways. Acetate is formed when ATP is generated from acetyl-CoA. This reaction is very important because anaerobic organisms lack an electron transport chain to create ATP by oxidation of NADH. When butyrate is formed from acetyl-CoA, NAD^+ is regenerated for use in oxidative steps in the degradation of energy sources. In *F. varium*, evidence for the pathway shown in Scheme-1.1 was collected by growing *F. varium* on a peptone medium supplemented with [1,2-$^{13}C_2$] acetate.[55] Using ^{13}C NMR spectroscopy, isotopically enriched butyrate was detected. The ^{13}C-^{13}C coupling pattern in the ^{13}C butyrate was indicative of the combination of two acetate units.[54,56]



Scheme-1.1: Metabolic relationship between acetate and butyrate.

Subsequently, a proteomics study of *F. varium* identified the enzymes used in the acetate-butyrate pathway.[57] It is likely that the acetate-butyrate pathway plays a key role in the metabolic processes of *F. varium*.

1.3 Isotopic Labeling and Determination of Metabolism using NMR Spectroscopy:

NMR (nuclear magnetic resonance) is a non-destructive and non-invasive technique that is valuable for the study of metabolism. NMR spectra provide quantitative and qualitative data for a variety of metabolites produced from numerous metabolic pathways in complex biological systems. The data gathered from NMR experiments allow structures to be assigned based on chemical shift, coupling pattern and coupling constant data. The main disadvantage of NMR spectroscopy is its modest sensitivity. The

concentration detection limit is around 10 μM, although increasing the scan limit, using a stronger magnetic field or doing experiments at a lower temperature can be used to improve sensitivity.[58]

With the improvement of NMR techniques, using substrates labeled with ^{13}C isotopes has become popular. With NMR spectroscopy the position of the isotope in the metabolic product is determined from chemical shifts, enrichment, ^{13}C-^{13}C coupling patterns and coupling constants.[59] Then the position of the label in a target molecule is correlated with a metabolic process starting with the initial substrate.

Isotopic labeling is an important technique used to establish a metabolic connection between compounds. Typically, a labeled substrate is supplied and metabolism by the bacterium leads to incorporation of isotopic labels into the metabolic products.

Over the past 30 years, the use of ^{13}C isotopic labeling in tandem with NMR spectroscopy has been greatly used to study bacterial metabolism.[60] In microbial growth cultures supplemented with an isotopically ^{13}C labeled substrate, the enrichments of ^{13}C found in the final products can be used to establish precursor-product relationships, and, in some instances to identify intermediates, for example from the metabolism of a ^{13}C labeled amino acid.[61,62]

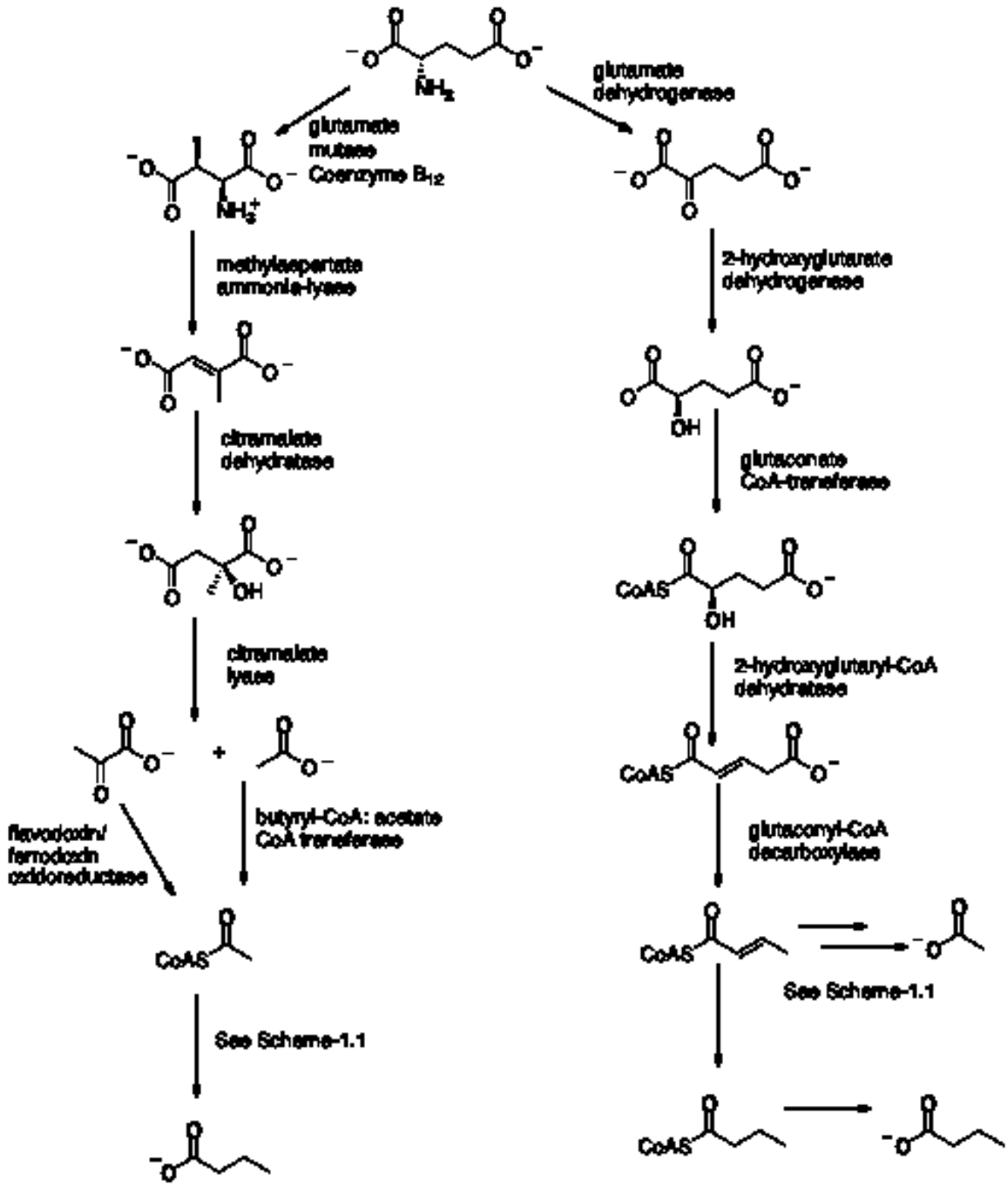
1.4 Glutamate Catabolism:

Glutamate is an important substrate for anaerobic bacteria and acts as a starting point for the biosynthesis of cellular constituents and as an energy source when degraded to acetate and butyrate.[22,63] Five chemically distinct pathways for glutamate catabolism in anaerobic bacteria have been documented,[64] but only three have been associated with *Fuscobacterium* species.[29,65] In *F. nucleatum*, the hydroxyglutarate pathway (Scheme-1.2) is well established[56,66] and is usually the reported pathway of glutamate catabolism.[22,57,67,68] An initial study attributed [1-^{14}C]glutamate catabolism to the hydroxyglutarate pathway in *F. varium*.[16] Later reports suggested that the methylaspartate pathway (Scheme-1.2) may play a factor, and presented conflicting evidence for the participation of the aminobutyrate pathway.[22,57,63,69]

Previous work using *F. varium* grown in peptone medium supplemented with [4-^{13}C]glutamate[55,69] has shown ^{13}C enrichment in acetate and equal enrichments at C1 and C3 of butyrate. This labeling pattern was consistent with the methylaspartate pathway. In a proteomic study, two enzymes were found for each of the methylaspartate and hydroxyglutarate pathways suggesting that *F. varium* has the capacity to form butyrate by two pathways.[69,70,71] In another study, genes for enzymes in both the methylaspartate and the hydroxyglutarate pathways were identified in the *F. varium* genome.[57,72] There was no evidence found for the aminobutyrate pathway.[54]

The pathway taken to synthesize butyrate by *F. varium* is influenced by the composition of the defined medium.[69] When grown in chemically defined medium, the transfer of the isotopic label from L-[4-^{13}C]glutamate to butyrate was consistent with the hydroxyglutarate pathway. When a small amount of vitamin B_{12} or cobalt chloride was

added to the chemically defined medium, isotopically labeling of butyrate at two sites was consistent with the methylaspartate pathway. Thus in the presence of cobalt, the methylaspartate pathway functions whereas in the absence of cobalt, the hydroxyglutarate pathway is utilized. These results are consistent with the requirement for a cobalt-containing coenzyme for the activation of glutamate mutase. While an earlier proteomic investigation[69] showed that glutamate mutase was expressed when *F. varium* was cultured on a defined medium, it was presumably inactive in the absence of the coenzyme. This information on the functioning of two pathways helps to show the diversity of *F. varium*'s metabolism.



Scheme-1.2: The methylaspartate (left) and hydroxyglutarate (right) pathways for the catabolism of L-glutamate to acetate and butyrate in *F. varium.*

1.5 Glucose Catabolism:

The ability of *F. varium* to derive energy from glucose was confirmed in a proteomics study.[57,72] When *F. varium* was grown on chemically defined minimal medium containing 1 mM amino acids and glucose, an increase of seven glycolytic enzymes was reported, indicating catabolism of glucose by the Embden-Meyerhof-Parnas (EMP) pathway (Scheme-1.3). All of the EMP enzymes and glycerol enzymes are encoded in the *F. varium* genome.[57] As a major energy source for *F. varium*, glucose yields two molecules each of ATP and NADH on conversion to pyruvate via the EMP pathway.[52,63] Pyruvrate can be reduced to lactate, which regenerates NAD^+. Alternatively, decarboxylation of pyruvate yields acetyl CoA, an intermediate of the acetate-butyrate pathway (Scheme-1.1). Acetyl CoA can be converted to acetyl phosphate for ATP generation or to butyrate via the acetate-butyrate pathway for regeneration of NAD^+.

A labeling experiment was carried out to determine the fermentation products produced directly from D-[U-$^{13}C_6$]glucose fed to *F. varium*.[58] ^1H and ^{13}C NMR spectroscopy were used to detect the isotopic labels.[58] Isotopic enrichments of butyrate, formate and lactate were established by the observation of intense ^{13}C satellite signals in the ^1H NMR spectrum of the lyophilized culture supernatant. The ^{13}C NMR resonances of butyrate showed ^{13}C-^{13}C coupling consistent with the three different enrichment patterns, shown in Figure-1.1.[68] The ^{13}C enrichments are consistent with the synthesis of butyrate by the combination of two acetate units (Scheme-1.1). The $^{13}C_2$-enriched butyrates demonstrate that unlabelled acetyl CoA is also formed, most likely from the amino acids present in the chemically defined medium.

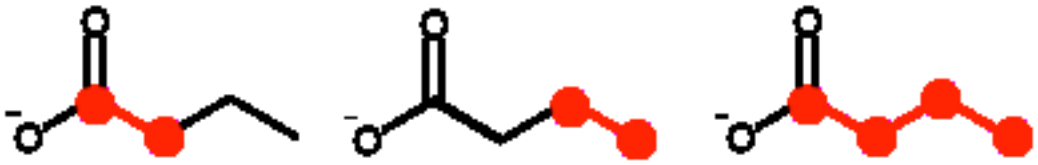
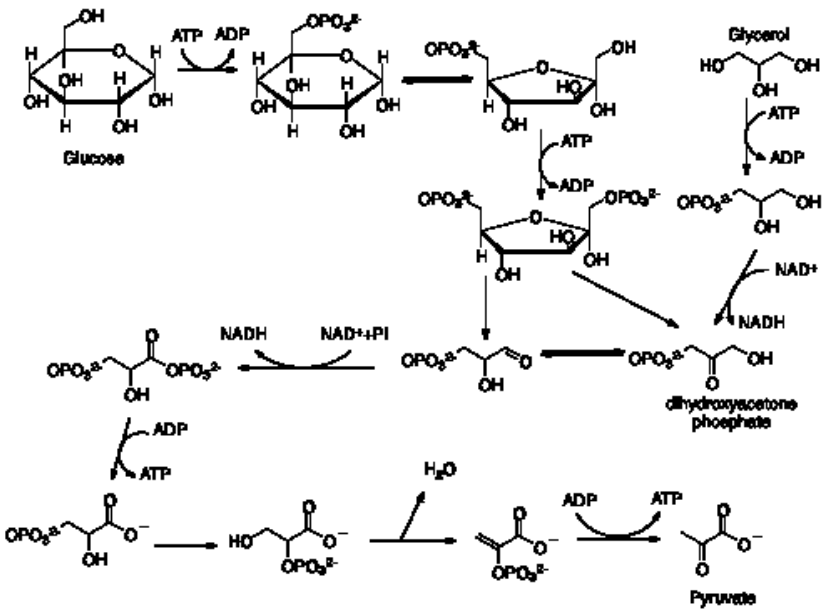


Figure 1.1: ^{13}C enrichment patterns of butyrate derived from D-[U-$^{13}C_6$]glucose.

The lack of ^{13}C incorporation from D-[U-$^{13}C_6$]glucose into propionate and succinate indicates that they are formed from other sources in the growth medium. Formation of propionate from threonine was subsequently shown,[58] but the origin of succinate has not been defined. While the labeling results establish glucose-product relationships, the ^{13}C enrichments of acetate, butyrate, formate, and lactate from D-[U-$^{13}C_6$]glucose were less than 100 % indicating that other medium components, such as amino acids, are catabolized to their fermentation products.

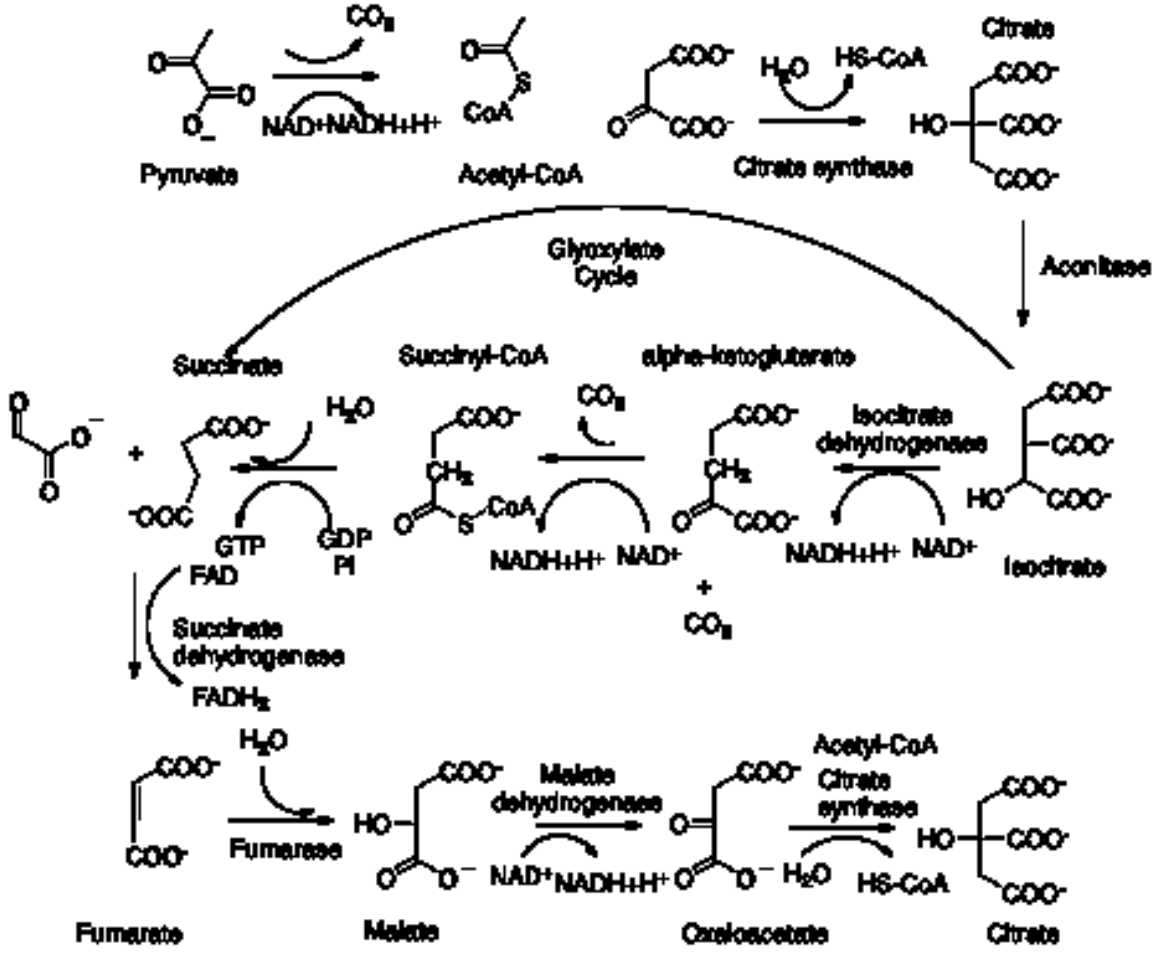


Scheme-1.3: Catabolism of glucose and glycerol to pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway in *F. varium*.

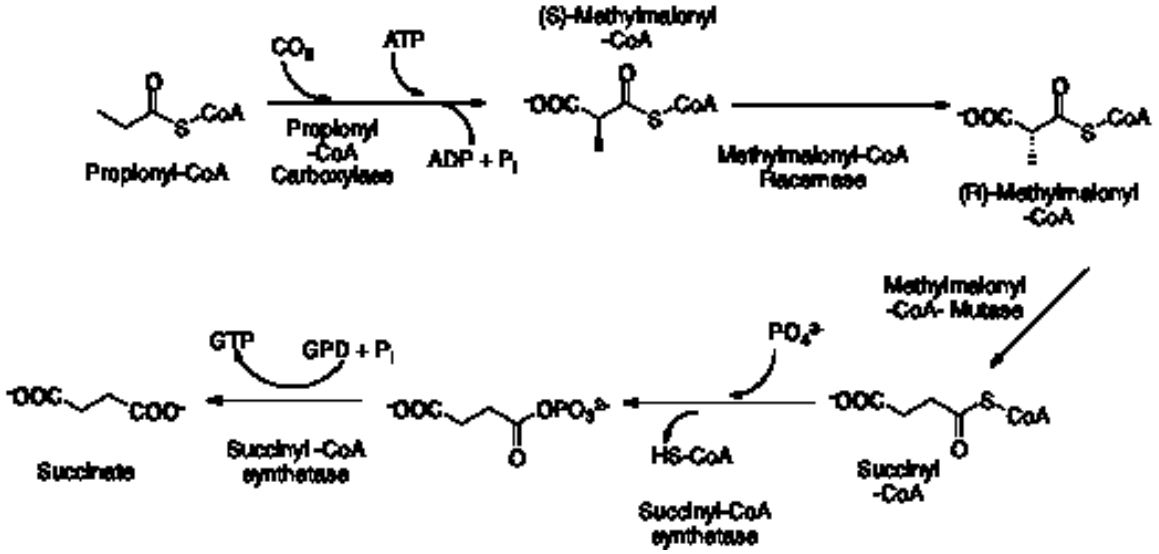
1.6 Succinate Formation:

Succinate has been identified as one of the renewable building block chemicals with the greatest technical feasibility and commercial potential and is normally formed as a byproduct of fermentation.[73] In *F. varium*, minor amounts of succinate are typically formed during fermentation of glucose in complex or defined culture medium.[42] Although a specific metabolic route to succinate in *F. varium* has not been identified, there are numerous possible precursors of succinate to consider, such as fumarate, malate, and oxaloacetate, which are known intermediates in the citric acid cycle (Scheme-1.4). Under anaerobic conditions the normal direction of the citric acid cycle is reversed to generate succinate as a reduction product from oxaloacetate. Alternatively, succinate could be formed from aspartate or by rearrangement of methylmalonyl CoA (Scheme-1.5). Methylmalonyl CoA is a metabolic intermediate formed by carboxylation of propionate or by degradation of amino acids or the pyrimidine thymine.

Succinic acid is a platform chemical used in the food, chemical, and pharmaceutical industries.[73] In 2004, succinic acid was identified by the US Department of energy as one of twelve chemicals that can be produced from biomass on a large scale as an alternative to petrochemical sources. A supply of bio-derived succinic acid would support the sustainable manufacture of higher value commodities, such as 1,4-butanediol, γ-butyrolactone, tetrahydrofuran and biodegradable polymers.[73,74,75,76,77]



Scheme-1.4: Citric Acid Cycle.



Scheme-1.5: Forming succinate from methylmalonyl CoA

Recently, large-scale fermentation processes have been implemented. The organisms used in the industrial bio-production of succinic acid are either natural producers or genetically modified nonproducting anaerobes.[78] The improvements made through genetic engineering include optimizing the succinate pathways, eliminating competing metabolic pathways that yield unwanted by-products, and introducting succinate pathways into nonproducing organisms.[77,79] Other modifications include the removal of the gene responsible for producing succinate dehydrogenase, which has made the aerobic production of succinate possible via a disrupted citric acid cycle (Scheme-1.4).[77,80] The reductive branch of the citric acid pathway (oxaloacetate → malate → fumarate → succinate) is utilized under anaerobic conditions.[77,79] In both circumstances, succinate may also be formed by the metabolism of acetyl-CoA via the glyoxylate cycle (i.e., cleavage of isocitrate to glyoxylate and succinate).

In an alternative approach, the typically low yield of succinate by the facultative anaerobe *Enterococcus faecalis* was boosted by the addition of fumarate to cultures using glycerol as a carbon source.[81,82,83] The approach was extended by using immobilized cells and flow systems.[81,82,83] The metabolism of glycerol yields dihydroxyacetone phosphate, an intermediate of the EMP pathway (Scheme-1.3). The subsequent conversion of dihydroxyacetone phosphate to pyruvate generates two NADH and a net gain of one ATP. The production of NADH provides the co-substrate needed for reduction of fumarate and regenerates NAD^+ needed for oxidation of glycerol. A further benefit to the use of glycerol is its availability in large quantity as a byproduct of biodiesel production and experiments have shown that glycerol is a viable co-substrate for succinate production.[81,82,83]

1.7 Thesis Goals:

The purpose of this study is to establish the presence of metabolic processes in *F. varium*. *F. varium* can catabolize glutamate to acetate and butyrate using either the hydroxyglutarate or methylaspartate pathway. Isotopic labeling will be used to distinguish between the two distinct pathways. A bond labeling approach is to be attempted using [$^{13}C_5$]glutamate to generate labeled metabolic products. The ^{13}C-^{13}C coupling maintained when bonds in glutamate are retained in the metabolic product will determine the pathway taken by glutamate. The appearance of three contiguous bonds (4 ^{13}C atoms) is predicted by the hydroxyglutarate pathway (glutamate→butyrate) whereas only two contiguous ^{13}C atoms would result from catabolism of glutamate by the methylaspartate pathway. This study should allow some insight into how glutamate is catabolized in an anearobic environment and whether or not an alternative pathway is taken.

The potential use of *F. varium* for the bioproduction of succinate will be explored. Initially, cultures of *F. varium* will be supplemented with potential precursors of succinate and examined by ^1H NMR spectroscopy to look for a build up of succinate. Enhanced succinate production will signify a metabolic connection and an indication of possible bioconversion. For a promising precursor, culture conditions will be optimized to promote the production of succinate. A detailed look into a range of energy sources to grow *F. varium* and to supply reducing equivalents, as well as growth conditions versus cell resuspensions will be investigated.

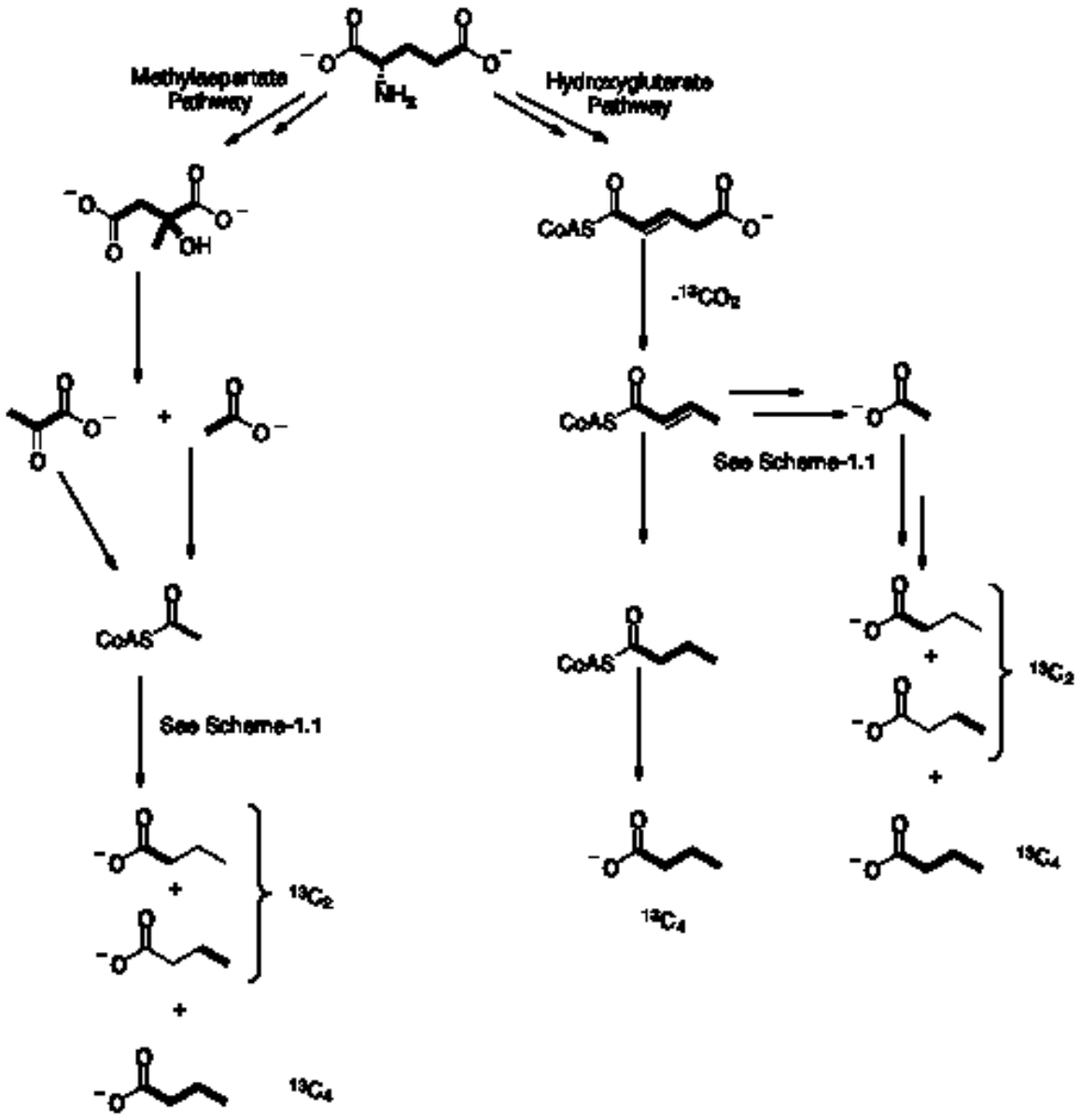
CHAPTER 2: PATHWAY DETERMINATION OF BUTYRATE IN ANAEROBIC BACTERIA USING UNIFORMLY LABELED GLUTAMATE AND ^{13}C NMR SPECTROSCOPY

2.1 Introduction:

As mentioned previously (Section-1.1), *F. varium* is an important bacterial species for colon health and beneficial colonic health effects are also associated with short chain aliphatic carboxylic acids.[39,63] These short chain aliphatic carboxylic acids include propionic acid and butyric acid. *F. varium* produces acetate and butyrate as major metabolic products when catabolizing glucose and amino acids and converts acetate to butyrate for coenzyme regeneration.[42]

Two possible pathways for the catabolism of glutamate in *F. varium* are known as the hydroxyglutarate pathway and the methylaspartate pathway (Section-1.4). In order to fully understand the metabolic processes for the synthesis of butyrate, the conditions that cause each pathway to occur must be understood. Previous work has shown that the hydroxyglutarate pathway is used when *F. varium* is cultured on a chemically defined minimal medium while the methylaspartate pathway functions when cobalt ions are available.[69] If a source of cobalt is introduced into the minimal medium, the methylaspartate pathway becomes more favorable, indicating that the glutamate pathway taken can be regulated and/or controlled by an environment factor. In order to identify which glutamate pathway is used in *F. varium*, in the formation of butyrate, the transfer of labeled L-[$^{13}C_5$]glutamate to butyrate was investigated in conjunction with carbon NMR spectroscopy to identify the distribution of isotopic label in fermentation products.

As shown in Scheme-2.1, the isotopic pattern in butyrate derived from L-[$^{13}C_5$]glutamate is specific to each pathway. In the methylaspartate pathway, glutamate is cleaved and butyrate is formed by a combination of labeled and unlabeled acetate units when the level of ^{13}C labeling is low. Therefore, the resulting butyrate will most likely contain two ^{13}C atoms. Also, the chance combination of two [$^{13}C_2$]acetate units is low, and the formation of [$^{13}C_4$]butyrate will be negligible. By contrast, the hydroxyglutarate pathway involves the formation of butyrate through the loss of CO_2.[69] If labeled [$^{13}C_5$]glutamate is degraded by this pathway, then [$^{13}C_4$]butyrate would be produced. Thus, the different labeling patterns found in butyrate will identify the pathway taken and allow the effect of culture conditions (e.g., cobalt availability) on glutamate catabolism to be investigated.



Scheme-2.1: Prediction of the incorporation of ^{13}C label into butyrate by catabolism of L-[$^{13}C_5$]glutamate.

2.2 Experimental:

2.21 Chemicals:

L-[U-$^{13}C_5$]glutamate (97-99 atom % ^{13}C) was purchased from Cambridge Isotope Laboratories (Andover, MA). Yeast extract and BHI agar were purchased from FlukaBiochemika (Buchs, Switzerland). Protease peptone and trypticase peptone were purchased from DIFCO laboratories (Detroit, MI). All other chemicals were obtained from Sigma-Aldrich (Oakville, ON).

2.22 Media:

BHI Agar: Agar plates were prepared from autoclaved (20 min, 120 ^oC) brain heart infusion agar (BHI; Fluka, Buchs, Switzerland) in deionized water (52.0 g/L).

Peptone Basal Medium: Peptone basal medium consisted of protease peptone (5.0 g/L), trypticase peptone (5.0 g/L), yeast extract (5.0 g/L), NaCl (5.0 g/L) and L-cysteine HCl (0.8 g/L) in deionized water. The medium was adjusted to pH 7.4 using 5 M NaOH and autoclaved (20 min, 120 ^oC). It was stored anaerobically until needed.

Peptone Medium: Peptone medium was prepared by adding D-glucose (5 %, m/v, 28 mM) to peptone basal medium. It was stored anaerobically until needed.

Amino Acid Medium: Modified chemically defined minimal medium consisted of 0.2 mM Group 1 amino acids (alanine, arginine, isoleucine, leucine, glycine, methionine, proline and valine), 1.5 mM Group 2 (tyrosine, tryptophan and phenylalanine) and 2 mM Group 3 amino acids (aspartate, asparagine, cysteine, glutamine, glutamate, histidine, lysine, serine, threonine). The solution of amino acids was readjusted to pH 7.4 using NaOH as needed and autoclaved (20 min, 120 ^oC).

Salt Medium: Salt medium consisting of 4.54 mM $(NH_4)_2SO_4$, 0.135 M $CaCl_2$,

0.0592 mM $MnSO_4$, 1.45 mM $MgSO_4$, 0.870 mM sodium citrate, 0.0360 mM $FeSO_4$, and 0.0615 μM Na_2MoO_4 was adjusted to pH 5.3 using NaOH and autoclaved (20 min, 120 ºC).

Vitamin Medium: The vitamin medium consisting of 9.19 μM 113 calcium D-pantothenate and 1.13 μM folic acid was adjusted to pH 4.5 using NaOH and HCl as needed and autoclaved (20 min, 120 ºC).

Basal Medium: Basal medium consisted of NaCl (5.00 g/L) and KH_2PO_4 (2.72 g/L) dissolved in water and was adjusted to pH 7.4 using 5 M NaOH.

Stock Glucose Solution: The stock glucose solution consisted of 43.3 g/L of glucose in basal medium.

Cobalt Solution: Consisted of 1 mg of cobalt (II) chloride hexahydrate dissolved in 1 mL of nanopure water and 0.1 mL was added to defined medium.

Defined Medium: The defined medium consisted of 250 mL of amino acid medium, 5 mL of salt medium, 5 mL of vitamin medium, 214 mL of basal medium, 21 mL of stock glucose solution, and 0.1 mL of cobalt solution. The final concentration of cobalt ions was 0.8 μM. All parts of the defined medium were stored together under a H_2:CO_2:N_2 (10:10:80 v/v) atmosphere except glucose, which was not combined until the peptone was ready to inoculate. The defined medium was then filtered through a non-pyrogenic sterile-R filter (0.2 μm) using a sterile 60 mL syringe.

PBS Acetate Solution: The PBS acetate solution consisted of 139 mM NaCl, 5.4 mM KCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, and 106 mM sodium acetate.

2.23 *F. varium* Stock Agar Culture:

A culture of *F. varium* (ATCC 8501, lyophilized from calf serum and stored at 4 ^{o}C) was spread on BHI agar. The agar plate was placed in an anaerobic jar containing palladium catalysts (Oxoid, low temperature anaerobic catalyst) and filled with a H_2:CO_2:N_2 (10:10:80 v/v) atmosphere. The container was incubated at 37 ^{o}C for 24 h and then stored at 4 ^{o}C. At biweekly intervals, a loopful of cells were transferred to fresh agar, incubated at 37 ^{o}C for 24 h and stored at 4 ^{o}C in anaerobic conditions.

2.24 Growth of *F. varium* in the Presence of Cobalt:

Peptone medium (10 mL) was inoculated with a loopful of cells from the stock agar plate and incubated anaerobically for 20 h at 37 ^{o}C. The peptone culture was centrifuged (5000 g, 10 min). The pelleted cells were washed with basal medium (5 mL) twice. After this, the cells were suspended in basal medium (5 mL) and transferred to defined medium with cobalt (500 mL).

The culture was then incubated for 16 h, and centrifuged (5000 g, 10 min). The supernatant was discarded and the cells (0.81 g damp mass) were resuspended in PBS acetate solution (40 mL). L-[$^{13}C_5$]Glutamic acid (7.0 mg) and L-glutamic acid (70 mg) were dissolved in 10 mL of PBS acetate solution and added to the cell suspension. The resuspended cells were incubated for 6 h. The supernatant was then collected and pH adjusted to 9.5 prior to freeze drying.

2.25 Derivatization of Butyrate and Acetate:

The freeze-dried sample (1.00 g) was mixed with 4-bromophenacyl bromide (1.70 g, 6.12 mmol) and dicyclohexano-18-crown-6 ether (1.30 g, 0.35 mmol) in acetonitrile (100 mL). TLC on silica gel with a solvent system of 100 % dichloromethane was used to

monitor the reaction. After refluxing for 6-8 h the mixture was cooled and filtered through a sintered glass funnel and rinsed with acetonitrile (4 x 10 mL). Rotary evaporation of the solvent yielded a yellow-orange oil.

2.26 Column Chromatography:

The oil was dissolved in a minimum amount of 30 % dichloromethane: cyclohexane and silica gel was added. The silica gel mixture was rotavapped until the silica was infused. The infused silica gel was added to a column prepared from approximately 70 g of silica gel (heated at 100 ºC overnight). A solvent system of 30 % dichloromethane: cyclohexane was used until *p*-bromophenacyl butyrate was eluted. Then the dichloromethane in the solvent system was increased by 5 % for every 100 mL used until *p*-bromophenacyl acetate eluted. If there was not good separation, a second column was run using 15 g of silica and a solvent system of 50 % chloroform: cyclohexane. Yield: *p*-bromophenacyl butyrate: 20 mg, *p*-bromophenacyl acetate: 468 mg.

The resulting derivatives of butyrate and acetate were analyzed using ^{1}H NMR spectroscopy to identify the product using the 300 MHz instrument and ^{13}C NMR was collected on the 500 MHz instrument to determine the^{13}C enrichments.

2.27 Growth of *F. varium* Without Cobalt:

The second experiment was done under the same reaction conditions and procedure except the cobalt solution was omitted from the defined medium.

2.28 Formation of Unlabeled *p*-Bromophenacyl Esters:

Sodium butyrate (0.0275 g, 0.25 mmol) and sodium acetate (0.451 g, 5.5 mmol) were combined with 4-bromophenacyl bromide (1.70 g, 6.12 mmol) and dicyclohexano-18-crown-6 ether (1.30 g, 0.35 mmol) in acetonitrile (100 mL). TLC on silica gel with a solvent system of 100 % dichloromethane was used to monitor the reaction. After refluxing for 6-8 h the mixture was cooled and filtered through a sintered glass funnel and rinsed with acetonitrile (4 x 10 mL). Rotary evaporation of the solvent yielded a yellow-orange oil. The separation was done by column chromatography as described above.

2.29 NMR Confirmation:

The ^{1}H and ^{13}C data collected for *p*-bromophenacyl acetate and butyrate shown below match published data.[56] The ^{13}C NMR spectra of the products formed using [$^{13}C_5$]glutamate are in the Results and Discussion.

p-Bromophenacyl acetate: ^{1}H NMR ([^{2}H]chloroform) δ: 2.21 (s, 3H,-CH_3), 5.26 (s, 2H, -CH_2-O-), 7.58 (apparent doublet, splitting, 8.8 Hz, 2H, aromatic) and 7.73 (apparent doublet, splitting, 8.8 Hz, 2H, aromatic); ^{13}C NMR ([^{2}H]chloroform) δ: 20.5 (CH_3, C-2), 65.7 (CH_2), 129.1, 129.2, 132.2, 132.8 (aromatic), 170.3 (C=O, ester, C-1), 191.4 (C=O, ketone).[56]

p-Bromophenacyl butyrate: ^{1}H NMR ([^{2}H]chloroform) δ: 0.99 (t, *J* = 7.2 Hz, 3H, -CH_3, 1.71, m, 2H, -*CH_2*-CH_3), 2.51 (t, *J* = 7.2 Hz, 2H, -(C=O)-CH_2-), 5.31 (s, 2H, -CH_2-O-), 7.65-7.60 (m, 2H, aromatic) and 7.80-7.76 (m, 2H, aromatic); ^{13}C NMR ([^{2}H]chloroform) δ: 13.7 (CH_3, C-4), 18.8 (*CH_2*-CH_3, C-3), 35.6 (*CH_2*-C=O, C-2), 65.5

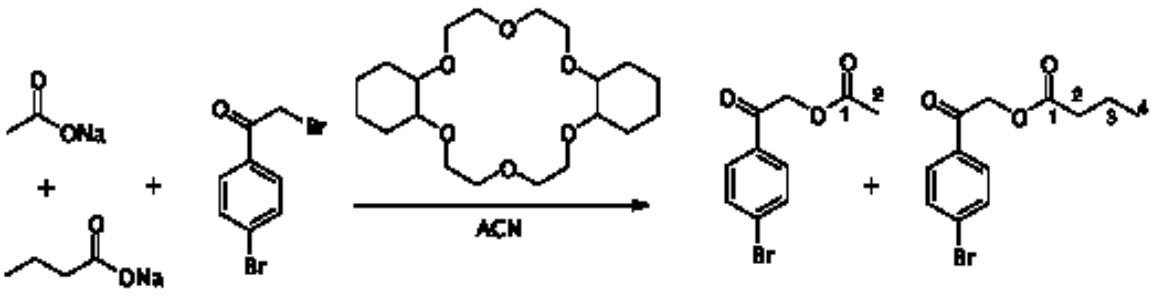
(CH_2-O), 129.2, 129.4, 132.3, 133.0 (aromatic), 173.2 (C=O, ester, C-1), 191.6 (C=O, ketone).

The ^{1}H NMR spectra (typically 32 scans) of the residues in $CDCl_3$ with 1 % TMS (15 mg/0.75 mL) were acquired on a Bruker AVANCE 300 MHz spectrometer using standard acquisition parameters. The data were processed using TopSpin software with line broadening of 0.3 Hz prior to Fourier transformation. The baseline was corrected manually with a polynomial function. Peaks were phase adjusted manually; chemical shifts were referenced to the TMS signal (δ 0.0). The ^{13}C NMR spectra analysis followed the same procedure: however, the spectra were collected on a Bruker AVANCE 500 MHz spectrmeter (^{13}C: 125 MHz, ~2000 scans).

2.3 Results and Discussion:

2.31 Incubation of *F. varium* with L-[$^{13}C_5$]Glutamate:

In separate experiments, *F. varium* was grown in defined medium and defined medium supplemented with cobalt chloride (0.001 mM). The *F. varium* cells were resuspended in PBS containing L-[$^{13}C_5$]glutamate and acetate (100 mM). The carboxylic acids formed by the resuspended cells were trapped as sodium salts and derivatized with *p*-bromophenacyl bromide[56] (Scheme-2.2). The resulting *p*-bromophenacyl esters were separated using column chromatography and analyzed by ^{13}C NMR spectroscopy.



Scheme 2.2: Synthesis of the *p*-bromophenacyl esters of acetate and butyrate.

2.32 NMR Spectra of *p*-Bromophenacyl Esters:

The ^{13}C NMR resonances assigned to the carbons in the acetyl group of the *p*-bromophenacyl acetate samples isolated from the L-[$^{13}C_5$]glutamate incubations are shown in Figure-2.1 and Figure-2.2. The other signals in the spectra corresponding to carbons derived from the *p*-bromophenacyl reagent appeared as singlets and have been omitted.

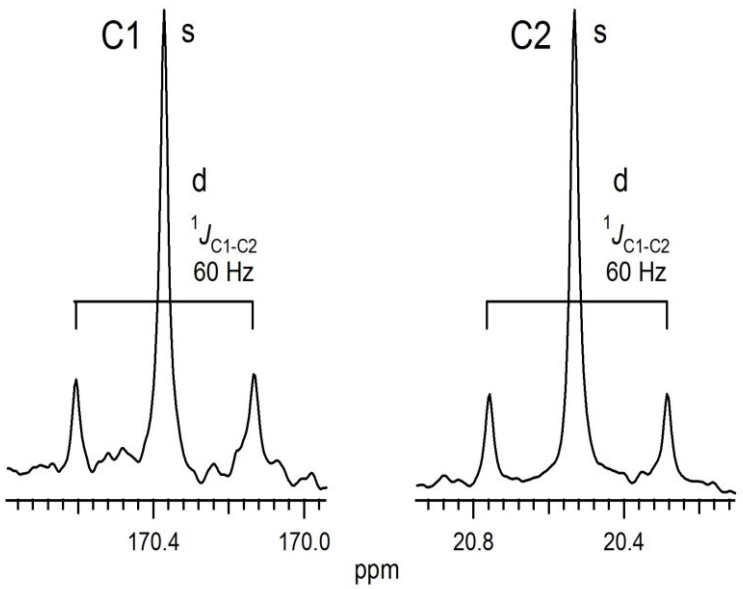


Figure-2.1: Partial ^{13}C NMR spectrum of *p*-bromophenacyl acetate (125 MHz, proton decoupled) showing ester carbonyl and methyl group resonances. The acetate was formed by *F. varium* cells grown on defined medium supplemented with Co^{2+} and incubated with L-[$^{15}C_5$]glutamate.

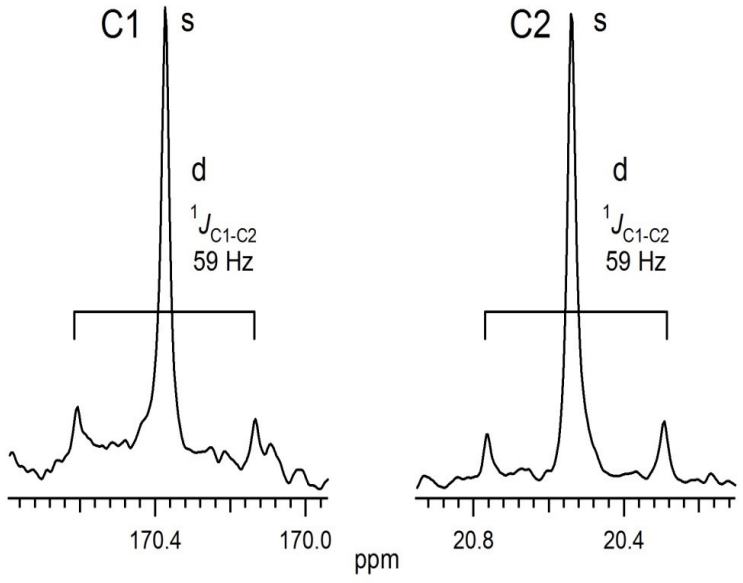


Figure-2.2: Partial ^{13}C NMR spectrum of *p*-bromophenacyl acetate showing the ester carbonyl and methyl group resonances. The acetate was formed by *F. varium* cells grown on defined medium (no Co^{2+}) and incubated with L-[$^{15}C_5$]glutamate.

In each ^{13}C NMR spectrum (Figure-2.1 and Figure-2.2), the ester carbonyl and the methyl group signals at 170.4 and 20.5 ppm, respectively,[56] appeared as a singlet flanked by a lower intensity doublet with a coupling constant of approximately 60 Hz. The singlet resonances arise from a ^{13}C nucleus bonded to ^{12}C, whereas the doublet signals demonstrate coupling to the adjacent ^{13}C nucleus. The observation of ^{13}C-^{13}C coupling in the acetyl group demonstrates the catabolism of L-[$^{13}C_5$]glutamate to [$^{13}C_2$]acetate. Also,^{13}C enrichments (Table-2.1) were calculated by dividing, the peak intensity for each carbon in the acetyl group by the average peak intensity for the carbons in the phenacyl moiety. These normalized intensities were divided by the normalized intensity calculated from the spectrum of a standard sample containing ^{13}C at natural abundance (approx. 1.1 %). The ^{13}C enrichment above natural abundance is equal to (ratio x 1.1) - 1.1 for the singlets and simply the ratio x 1.1 for the doublets. The ^{13}C enrichment calculated for the *p*-bromophenacyl acetates isolated from the incubations with L-[$^{13}C_5$]glutamate (Table-2.1) indicated no enrichment above natural abundance for the carbons giving rise to the singlet resonances and small enrichment for the $^{13}C_2$ isotopologue. Thus single atoms of ^{13}C were not transferred from L-[$^{13}C_5$]glutamate to acetate, only acetate containing two continuous ^{13}C atoms in glutamate were formed.

Table-2.1: ^{13}C enrichments (%) of the acetyl carbons in *p*-bromophenacyl acetate derived from L-[$^{13}C_5$]glutamate.

Pattern of ^{13}C Enrichment	Defined Medium (+Co^{2+})		Defined Medium (no Co^{2+})	
	C1	C2	C1	C2
^{13}C	0.13	0.075	0.086	-0.033
$^{13}C_2$	0.49	0.44	0.24	0.23

The ^{13}C resonances assigned to the butyryl group in the *p*-bromophenacyl bromide isolated from the incubation of L-[$^{13}C_5$]glutamate with *F. varium* cells grown on defined medium supplemented with cobalt chloride are shown in Figure-2.3. Again, signals corresponding to the carbons derived from the *p*-bromophenacyl acetate reagent appeared as singlets and have been omitted.

The resonances assigned to the carbons in the butyryl group[71] appeared as singlets flanked by lower intensity doublets. The magnitudes of the coupling constants (57 Hz and 34 Hz) were consistent with one-bond couplings between C1-C2 and C3-C4. The ^{13}C enrichments determined for the carbons in the butyryl group in *p*-bromophenacyl butyrate (Table-2.2) indicated that enrichments above natural abundance were only due to the incorporation of $^{13}C_2$ units.

Table-2.2: ^{13}C enrichments (%) of the butyryl carbons in *p*-bromophenacyl butyrate derived from L-[$^{13}C_5$]glutamate incubated with *F. varium* cells grown on defined medium supplemented with cobalt chloride.

Pattern of ^{13}C Enrichment	C1	C2	C1	C2
^{13}C	-0.18	0.035	-0.19	0.025
$^{13}C_2$	0.24	0.41	0.31	0.43

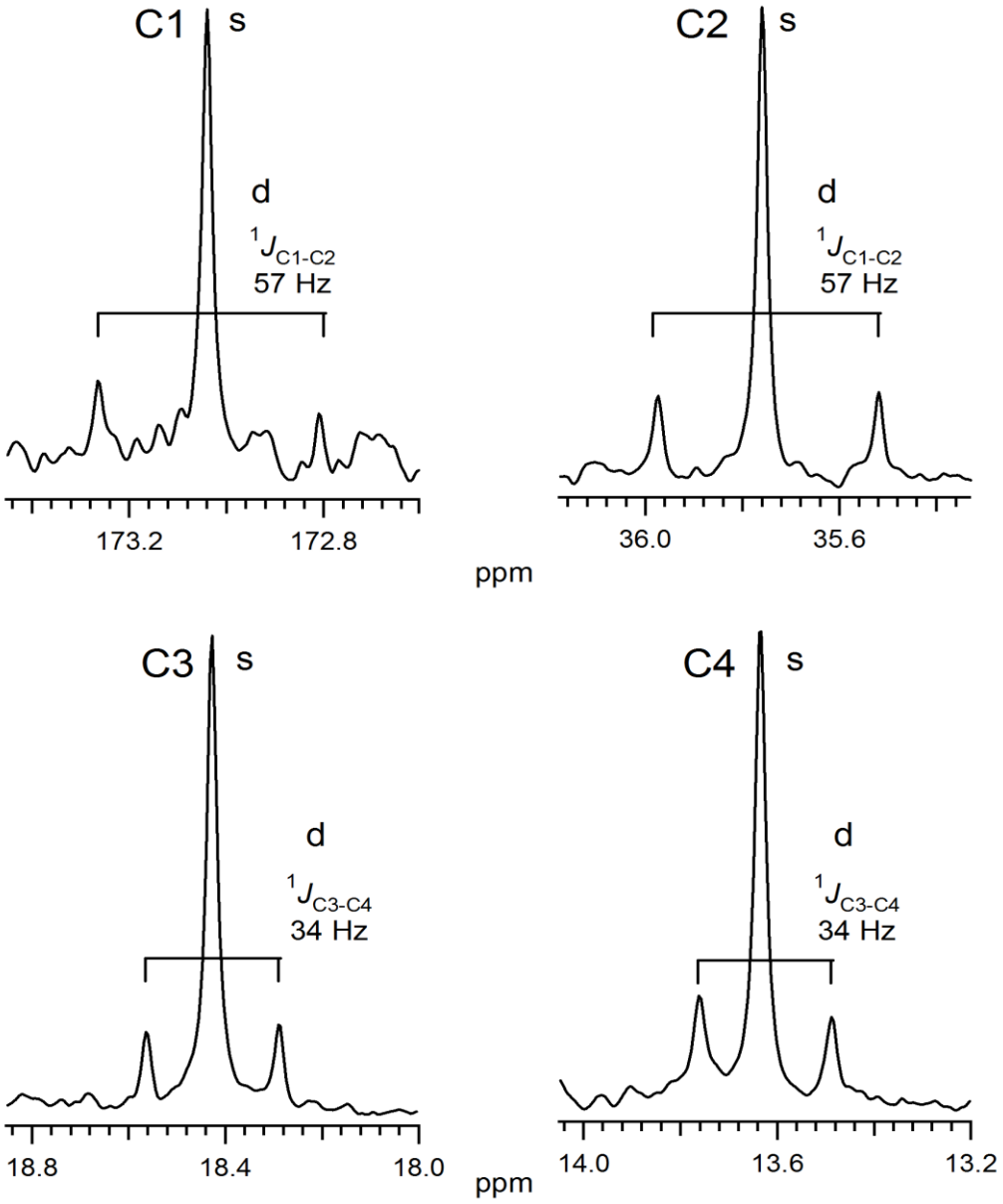


Figure-2.3: Partial ^{13}C NMR spectrum of *p*-bromophenacyl butyrate (125 MHz, proton decoupled) showing the resonances of the carbons derived from the butyryl group. The butyrate was produced by *F. varium* cells grown on defined medium supplemented with Co^{2+} and incubated with L-[$^{13}C_5$]glutamate.

The couplings between C1-C2 and C3-C4 (Figure-2.3) are consistent with the condensation of [$^{13}C_2$]acetyl-CoA with unlabelled acetyl-CoA to form [$^{13}C_2$]butyryl-CoA (Scheme-1.1). The coupling pattern and matching coupling constants, as well as the ^{13}C enrichments demonstrated catabolism of L-[$^{13}C_5$]glutamate with transfer of two contiguous carbons to butyrate. The $^{13}C_2$ enrichment in butyrate (Table 2.2) was similar to that of acetate isolated from the same experiment (Table-2.1). Thus the sequence L-[$^{13}C_5$]glutamate → [$^{13}C_2$]acetate → [$^{13}C_2$]butyrate (Scheme-2.1) is supported by the isotopic incorporations and was reported previously for *F. varium* grown on undefined medium.[55,71] Since two acetyl-CoA are combined; the label has an equal chance of being located at C1/C2 and C3/C4 in the product butyryl-CoA. The combination of two [$^{13}C_2$]acetyl-CoA is unlikely because low ^{13}C enrichments (Table-2.1) were ensured by the high concentration of unlabelled acetate and the unlabelled L-glutamate supplied with L-[$^{13}C_5$]glutamate. Indeed, no coupling between C2 and C3 was observed (Figure-2.3). Overall, these results are most consistent with catabolism of glutamate by the methylaspartate pathway.

In a parallel experiment, L-[$^{13}C_5$]glutamate was catabolized by *F. varium* cells grown on defined minimal medium (no Co^{2+}). The ^{13}C NMR resonances assigned to the carbons in the butyryl group of *p*-bromphenacyl butyrate are shown in Figure-2.4. The singlet resonances at 173.0 and 13.6 ppm corresponding to ester carbonyl and methyl group carbons, respectively, are accompanied by less intense doublets. The coupling constants of 57 (C1) and 35 (C4) Hz are consistent with C1-C2 and C3-C4 coupling as described above. Thus [1,2-$^{13}C_2$]- and [3,4-$^{13}C_2$]butyrate were also formed in this experiment.

While the singlet at 35.8 ppm is accompanied by a doublet resonance (J = 57 Hz) confirming the coupling seen at C1, a doublet of doublets pattern is also evident. The doublet of doublets arises from simultaneous coupling of C2 to C1 (57 Hz) and C3 (34 Hz). Similarly, the singlet at 18.4 ppm (C3) is accompanied by a less intense doublet (J = 35 Hz) confirming coupling to the adjacent C4. The doublet resonance is flanked by two other signals of similar intensity. The separation of the signals (68 Hz) corresponds to the sum of the coupling constants for C2-C3 and C3-C4 coupling. The inner signals of these coupling patterns are obscured by the singlet resonance. The broadened outer signals are indicative of two-bond coupling.

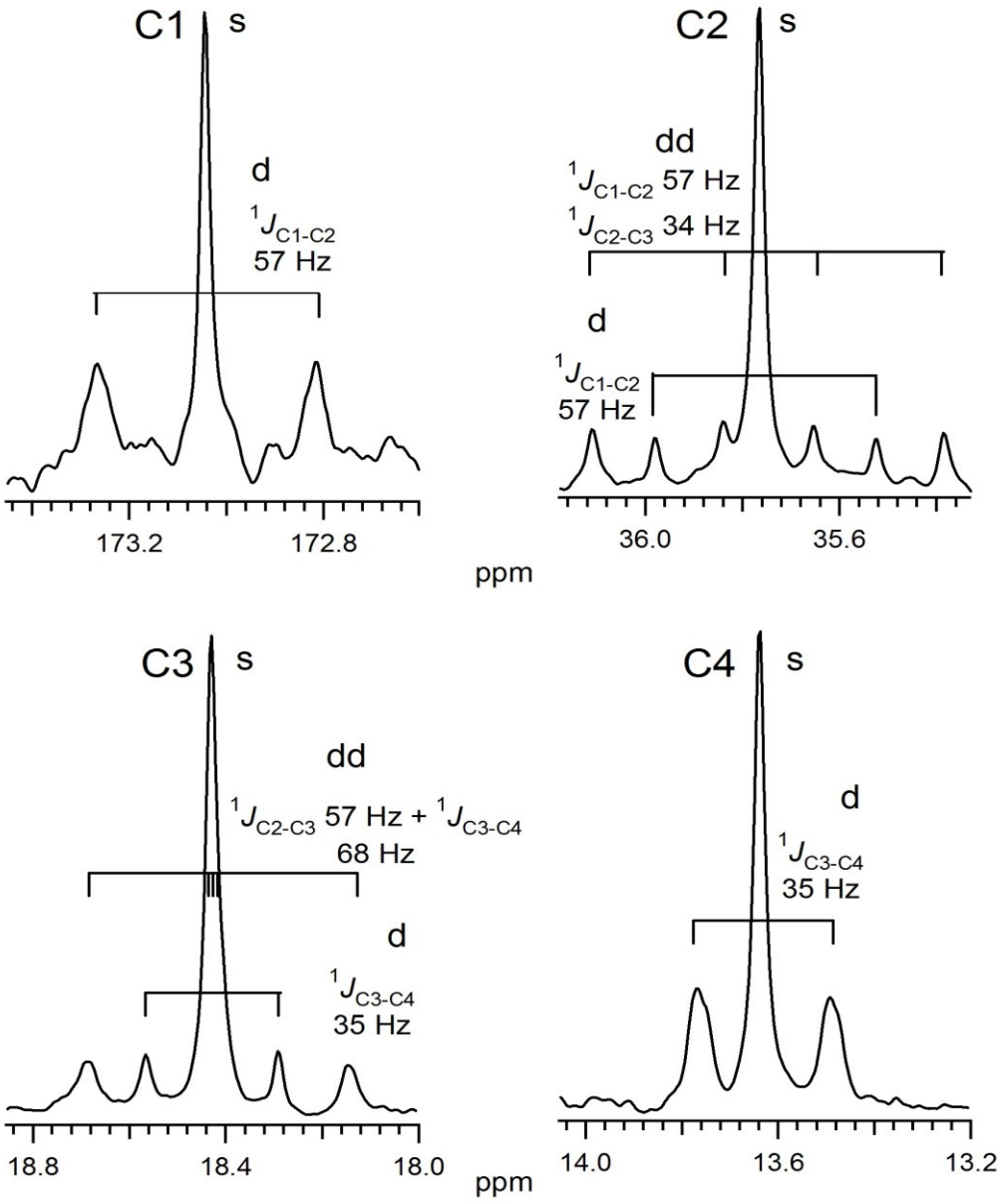


Figure-2.4: Partial ^{13}C NMR spectrum of *p*-bromophenacyl butyrate (125 MHz, proton decoupled) showing the resonances of the carbons derived from the butyryl group. The butyrate was produced by *F. varium* cells grown on defined medium (no Co^{2+}) and incubated with L-[$^{13}C_5$]glutamate.

The one-bond, ^{13}C-^{13}C coupling of C2 with both C1 and C3, as well as coupling of C3 to both C2 and C4, demonstrates the formation of [$^{13}C_4$]butyrate. The broadened doublet resonances for C1 and C4 indicating long-range, ^{13}C-^{13}C coupling are also consistent with $^{13}C_4$ enrichment. The $^{13}C_4$ enrichment in butyrate (ca. 0.58 %, Table-2.3) is higher than that measured for [$^{13}C_2$]butyrate (ca. 0.27 %) and [$^{13}C_2$]acetates (ca. 0.24 %, Table-2.1). Moreover, the $^{13}C_4$ enrichment is about 1000 times higher than that expected for the chance of recombination of two [$^{13}C_2$]acetate units (0.0006 % from 0.0024 x 0.0024 x 100). Thus the isotopic labeling results strongly support the transfer of four contiguous carbons from glutamate to butyrate and the sequence L-[$^{13}C_5$]glutamate → [$^{13}C_4$]butyrate → [$^{13}C_2$]acetate → [$^{13}C_2$]butyrate (Scheme-2.1). The transfer of four contiguous carbons from L-[$^{13}C_5$]glutamate to butyrate is characteristic of the hydroxyglutarate pathway (Scheme-2.1).

From the ^{13}C spectra (Figure-2.3 and Figure-2.4), there is clearly a difference in the ^{13}C enrichments of butyrate isolated from the culture grown with cobalt and the culture grown without cobalt. The calculations show that the $^{13}C_4$ enrichments in butyrate are not due to chance. The only difference between the two experiments was the addition of cobalt ions, and this one variation had a major effect on the route taken for the catabolism of glutamate in *F. varium*.

Table-2.3: ^{13}C enrichment (%) in the butyrate carbons in *p*-bromophenacyl butyrate derived from L-[$^{13}C_5$]glutamate incubated with *F. varium* cells grown on defined medium (no Co^{2+}).

Pattern of ^{13}C Enrichment	C1	C2	C3	C4	Average
^{13}C	0.0047	0.28	-0.26	0.22	0.06
$^{13}C_2$	0.19	0.28	0.28	0.34	0.27
$^{13}C_4$	0.58	0.69	0.47	0.58	0.58

2.33 Pathways Used for Butyrate Production:

The labeling results obtained in the two [$^{13}C_5$]glutamate experiments provided evidence for two pathways of glutamate catabolism in *F. varium.*[69] The distinct ^{13}C-^{13}C coupling patterns observed in butyrate (Figure-2.3 and Figure-2.4) were attributed to the methyl aspartate and hydroxyglutarate pathways. Moreover, the results clearly showed that the pathway utilized for glutamate catabolism in *F. varium* is strongly influenced by the supply of cobalt ions.

When a small amount of the cobalt ions were available, the methylaspartate pathway (Scheme-1.2) was the dominant route for glutamate catabolism. The cobalt is a component of coenzyme B_{12}, an essential cofactor for the enzyme glutamate mutase that catalyzes the first step in the methylaspartate pathway. Under these circumstances, glutamate is broken down to acetate. The large concentration of unlabeled acetate added with [$^{13}C_5$]glutamate diluted the [$^{13}C_2$]acetate formed from [$^{13}C_5$]glutamate and reduced the chances of two labeled acetates combining to form [$^{13}C_4$]butyrate. In fact, the lack of C2-C3 coupling (Figure-2.3) indicates no significant formation of [$^{13}C_4$]butyrate. These

results are consistent with isotopic evidence for the methylaspartate pathway in *F. varium* indicated by previous reports[55,69,72,84]

When cobalt is not available, *F. varium* is unable to synthesize an active form of coenzyme B_{12}. Without this essential cofactor, the first enzyme in the methylaspartate pathway is catalytically inactive, and the pathway is unable to function. Under these conditions, labels from [$^{13}C_5$]glutamate were transferred to butyrate as a contiguous unit of four carbon atoms signified by extensive ^{13}C-^{13}C coupling (Figure-2.4), and glutamate was mostly catabolized by the hydroxyglutarate pathway (Scheme-2.1). Previously, degradation of glutamate by the hydroxyglutarate pathway was supported by the incorporation of a single ^{13}C label into mainly one position in butyrate,[69] by nonspecific enzyme assays in crude cell extracts,[65] and the identification of genes for each enzyme in the hydroxyglutarate pathway in the genome of *F. varium*.[72] The current results add to the accumulated evidence for a distinct second pathway of glutamate catabolism in *F. varium*.

To a smaller extent, ^{13}C label was also located in [$^{13}C_2$]acetate and [$^{13}C_2$]butyrate indicating breakdown of [$^{13}C_4$]butyrate to [$^{13}C_2$]acetyl-CoA and recombination of labeled and unlabelled acetate units to form [$^{13}C_2$]butyrate (Scheme-2.1). Alternatively, a small amount of [$^{13}C_2$]acetate could have been formed by catabolism of [$^{13}C_5$]glutamate by the methylaspartate pathway.

The bond labeling approach used in this investigation gave complementary information to previous studies[56,69] by demonstrating the incorporation of substructures of precursors that were larger than a single atom. The chemical shifts of the ^{13}C coupled resonances were different than those of the signals due to ^{13}C at natural abundance. This

made the incorporation of contiguous ^{13}C atoms readily apparent in the ^{13}C NMR spectra, and the sensitive detection of signals over background noise. When single ^{13}C atoms are incorporated, they must be detected by increased peak height of signals from ^{13}C of natural abundance and not the appearance of new distinct signals in the ^{13}C NMR spectrum.

The results of these experiments reinforced the presence of two distinct pathways for glutamate catabolism in *F. varium*.[69,72] The results also showed that the environment has a major influence on the pathway utilized. The capacity to degrade glutamate by two pathways suggests that glutamate catabolism is an important aspect of *F. varium*'s metabolism and its ability to adapt to different environments.

CHAPTER 3: PRODUCTION OF SUCCINIC ACID FROM FUMARIC ACID AND VARIOUS CARBON SOURCES USING *F. VARIUM* AND RESUSPENSION CONDITIONS

3.1 Introduction:

As mentioned previously (Section-1.6), succinate has been identified as one of several metabolic products formed during fermentation of glucose by *F. varium*.[42] Intermediates in the citric acid cycle (Scheme-1.4) are typical precursors of succinate, but other precursors are possible such as aspartate or methylmalonyl CoA (Scheme-1.5). The numerous possible precursors means succinic acid may be formed in a variety of metabolic pathways. However, the specific metabolic routes leading to succinate production in *F. varium* have not been identified.

As a platform chemical, succinic acid has many uses in the food, chemical, and pharmaceutical industries and has recently gained heavy interest in its bioproduction. The production of bio-derived succinic acid is needed to support sustainable manufacturing of other commodities,[73-77] and the recent interest in bio-succinic acid has led to the implementation of large-scale production facilities. In these processes, parts of the citric acid cycle are used to form succinate under both aerobic and anaerobic conditions.[77-79]

Like *F. varium*, the facultative anaerobe *Enterococcus faecalis* produces succinate in low yield. However, recent experiments have shown that the addition of a metabolic precursor of succinate, namely fumarate raised the level of succinate production.[81,82,83] In these cultures, the NADH needed for the reduction of fumarate was generated by oxidation of glycerol and a subsequent step in the EMP pathway (Scheme-1.3). In

general, the production of succinate from fumarate requires a supply of reducing agent typically supplied by catabolism of a carbon source.

The goal of this investigation is to investigate whether succinate production is increased by the addition of a potential precursor and to systemically adjust culture conditions to optimize the yield of succinate. This will be a step toward the development of an alternative process of the production of biosuccinic acid and serve as a guide for future genetic manipulation to optimize succinic production by *F. varium*

3.2 Experimental:

3.21 General Methods and Microorganism:

Bacterial transfers and centrifugations were performed in air; cultures were incubated and stored in anaerobic jars containing a palladium catalyst (Oxoid Canada, Nepean, ON) and an atmosphere of H_2:CO_2:N_2 (10:10:80 v/v/v) established by three evacuation/pressurization cycles. Culture media were autoclaved at 121°C for 20 min. *Fusobacterium varium* ATCC 8501 was maintained on brain heart infusion (BHI) agar (Fluka, Buchs, Switzerland) under anaerobic conditions; a freshly inoculated BHI agar plate was prepared biweekly, incubated at 37°C for 24 h, and then stored at 4°C.

Peptone medium[42] containing proteose peptone, trypticase peptone, yeast extract (each at 5 g L^{-1}), NaCl (5 g L^{-1}, 86 mM), L-cysteine HCl (0.8 g L^{-1}) and varying concentrations of a carbon source (glucose (5.4 g L^{-1}, 30 mM), glycerol (2.7 g L^{-1}, 30 mM) or sorbitol (5.4 g L^{-1}, 30 mM)) was adjusted to pH 7.4 and autoclaved. Starter cultures in peptone medium (30 mM glucose, 30 mM glycerol or 30 mM sorbitol) were inoculated with a colony of *F. varium* from BHI agar and incubated at 37°C under anaerobic conditions for 24 h.

Optical densities at 660 nm (OD_{660}) were measured in 1-cm cuvettes using an Agilent 8453 UV-Vis Spectrophotometer, and cells were separated from liquid cultures by centrifugation (8000 *g*, 20°C, 10 min). Cell densities wew based on the mass of damp cells.

The reproducibility of the experiments was established by replicate measurements collected in several initial experiments. With physical limitations on the capacity to conduct incubations under anaerobic conditions and a lengthy five-day time frame for

each experiment, the results collected for other experiments were based on single determinations.

3.22 Growth cultures:

Peptone medium (30 mM glucose, 30 mM glycerol or 30 mM sorbitol) was inoculated (1% v/v) from a starter culture (30 mM glucose, 30 mM glycerol or 30 mM sorbitol) and incubated anaerobically at 37°C. When peptone medium was supplemented with a potential precursor (20 or 40 mM) to evaluate the effect on succinate production, culture supernatants were collected after the incubation period (typically 14 h), and processed for ^{1}H NMR analysis as described below. *F. varium* cells for suspension experiments were harvested from peptone medium (normally 30 mM glucose; 40 mM fumarate) after incubation for 8 h (typically) and washed with phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.4).

3.23 Suspension experiments:

Washed *F. varium* cells from the growth culture (typically 200 mL) were suspended in an autoclaved solution (typically 10 mL) containing fumarate and a carbon source (glucose, glycerol, or sorbitol) at various concentrations and adjusted to pH 7.4. After incubation under anaerobic conditions for 3–24 h, the cells were separated by centrifugation, and the supernatants were processed for ^{1}H NMR analysis as described below.

3.24 Ethanol Extraction:

After the supernatant was collected from the suspension experiment, the cells were resuspended in 10 mL of 70 % ethanol or 10 mL of 70 % acidified ethanol adjusted to pH ~1.0. The new ethanol suspensions were stirred for 1 h, centrifuged and the supernatant collected. Rotary evaporation of the supernatant led to a colourless solid.

3.25 ^{1}H NMR and MS analysis:

After the addition of L-tartaric acid (20 mg/10 mL supernatant), supernatants from growth cultures and cell suspensions were adjusted to pH 9.5 by addition of 5 M NaOH and lyophilized. Lyophilized residues dissolved in methanol/water (1:1 v/v; 1 mg mL^{-1}) were analyzed by flow injection (20 μL min^{-1}) using electrospray ionization (ESI, negative mode) and a Thermo-Finnigan LCQ Duo mass spectrometer.[85]

^{1}H NMR spectra (typically 16 scans) of the lyophilized residues in D_2O (15 mg/0.75 mL) were acquired on a Bruker AVANCE 300 MHz spectrometer using standard acquisition parameters, except for a 10-s delay between pulses.[69] The data were processed using TopSpin software with line broadening of 0.3 Hz prior to Fourier transformation. The baseline was corrected manually with a polynomial function. Peaks were phase adjusted manually; chemical shifts were referenced to the residual water signal (δ 4.80). Concentrations were calculated from the integrated peak areas of metabolic products and residual substrates relative to that of a known concentration of the internal standard L-tartrate (δ 4.28, s, 2H). Quantitative data for acetate (δ 1.86, s, 3H), lactate (δ 1.28, d (J = 7 Hz), 3H), fumarate (δ 6.47, s, 2H) and succinate (δ 2.35, s, 2H)

were obtained from the area of one representative signal, whereas the peak areas of doublets at δ 5.19 (J = 4 Hz, α-anomer) and 4.61 (J = 8 Hz, β-anomer) were combined to determine the concentration of glucose.

3.3 Results and Discussion:

3.31 Precursor-directed Accumulation of Succinate:

Typically, succinate production of less than 3 mM was observed when *F. varium* was grown on peptone (30 mM glucose) medium (Table 3.1). To investigate whether the minor production of succinate was limited by the shortage of a metabolic precursor, *F. varium* was grown on peptone medium supplemented with a compound related to succinate metabolism in bacteria. Abundant growth of *F. varium* was attained in each culture (Tables 3.1 and 3.2), and extensive utilization of glucose with accumulation of acetate, lactate, and small amounts of formate was evident in the ^{1}H NMR spectra of the lyophilized culture supernatants (Figure 3.1). Accumulation of succinate (Tables 3.1 and 3.2) was not affected by the addition of precursors of methylmalonyl-CoA,[86] γ-aminobutyrate (GABA),[87] and several intermediates of the citric acid and glyoxylate cycles.[88] Increased accumulation of succinate was only found in the peptone culture supplemented with fumarate. An important note is that compounds one metabolic step away from fumarate (L-malate and L-aspartate) were ineffective in stimulating succinate production, as well as the addition of tartrate stereoisomers (Table 3.2)[89] or bicarbonate (Table-3.3)[90] to encourage the formation of oxaloacetate. ESI(–)MS analysis of the lyophilized culture supernatants detected ions corresponding to fermentation products (acetate, lactate, and succinate) and unmetabolized substrate, supplied as a potential precursor of succinate.

Table-3.1: Succinate production in growth cultures supplemented with potential precursors (20 or 40 mM) associated with methylmalonyl-CoA, which yields succinate upon rearrangement, and γ-aminobutyrate. Products and residual substrates in depleted peptone medium (initially 30 mM glucose) at 14 h were determined by ^{1}H NMR spectroscopy.

Potential Precursor	Conc. (mM)	OD_{660}	Residual Substrate (mM)		Fermentation Product (mM)		
			Glucose	Potential Precursor	Acetate	Lactate	Succinate
None[a]	na	1.5 ± 0.1	nd	na	6 ± 2	29 ± 6	2.3 ± 0.6
Propionate	20	1.9	nd	27[b]	4	33	nd
	40	1.9	nd	15	5	31	nd
3-Amino-isobutyrate	20	1.5	nd	21	5	28	nd
	40	1.4	nd	16[b]	6	34	nd
Thymine	20	1.1	nd	23	4	29	nd
	40	1.7	nd	15	5	35	nd
Isoleucine	20	1.5	nd	24[b]	14	30	nd
	40	1.4	nd	28	6	28	nd
Valine	20	1.1	nd	22[b]	5	28	nd
	40	1.3	nd	20	6	28	nd
γ-Aminobutyrate	20	1.5	nd	27[b]	4	27	nd
	40	1.4	nd	23	0.5	37	nd

na = not applicable; nd = not detected; [a] average (3 determinations) ± standard deviation; [b] detected by ESI-MS along with lactate (major ion) and acetate (minor ion).

Table-3.2: Succinate production in growth cultures supplemented with potential precursors (20 or 40 mM) associated with the citric acid and glyoxylate cycles. Products and residual substrates in depleted peptone medium (initially 30 mM glucose) at 14 h were determined by ^{1}H NMR spectroscopy.

Potential Precursor	Conc. (mM)	OD_{660}	Residual Substrate (mM)		Fermentation Product (mM)		
			Glucose	Potential Precursor	Acetate	Lactate	Succinate
None[a]	na	1.5 ± 0.1	nd	na	6 ± 2	29 ± 6	2.3 ± 0.6
Citrate[b]	20	1.8	nd	21 ± 3[c]	6 ± 1	38 ± 6	nd
	40	1.4	nd	21 ± 5	4 ± 1	34 ± 3	nd
α-Ketoglutarate	20	1.8	nd	16	6	8	1
	40	1.6	nd	8	8	24	nd
Fumarate[d]	20	2.2 ± 0.3	nd	0.3 ± 0.6	11 ± 2	30 ± 5	16 ± 2
	40	2.5 ± 0.3	nd	1.2 ± 1.4	20 ± 2	27 ± 7	30 ± 1
L-Aspartate[b]	20	1.7 ± 0.1	nd	27 ± 1	4.3 ± 0.1	33 ± 1	1.9 ± 0.2
	40	1.8 ± 0.1	nd	40 ± 1	6 ± 1	41 ± 4	nd
Glyoxylate	20	1.2	nd	nd	6	29	nd
	40	1.3	nd	nd	5	32	nd
L-Malate[b]	20	1.8 ± 0.1	nd	24 ± 3	5.5 ± 0.7	49 ± 12	3.4 ± 0.5
	40	1.9 ± 0.5	nd	45 ± 1	8 ± 1	43 ± 14	0.8 ± 0.3
Oxaloacetate	20	1.7	nd	nd	16	31	2.6
	40	2.9	nd	nd	19	53	2.7
meso-Tartrate	20	2.26	nd	20[e]	6	38	nd
	40	2.39	nd	40[e]	3	21	nd
L-Tartrate	20	2.49	nd	20[e]	5	28	nd
	40	2.56	nd	40[e]	3	20	0.8
DL-Tartrate	20	2.11	nd	20[e]	5	29	nd
	40	2.30	nd	40[e]	3	17	nd

na = not applicable; nd = not detected; [a] average (3 determinations) ± standard deviation; [b] average (2 determinations) ± ½ difference between the determinations; [c] detected by ESI-MS; [d] average (4 (20 mM) and 5 (40 mM) determinations) ± standard deviation; [e] initial concentration used as a reference for estimating the concentration of other components.

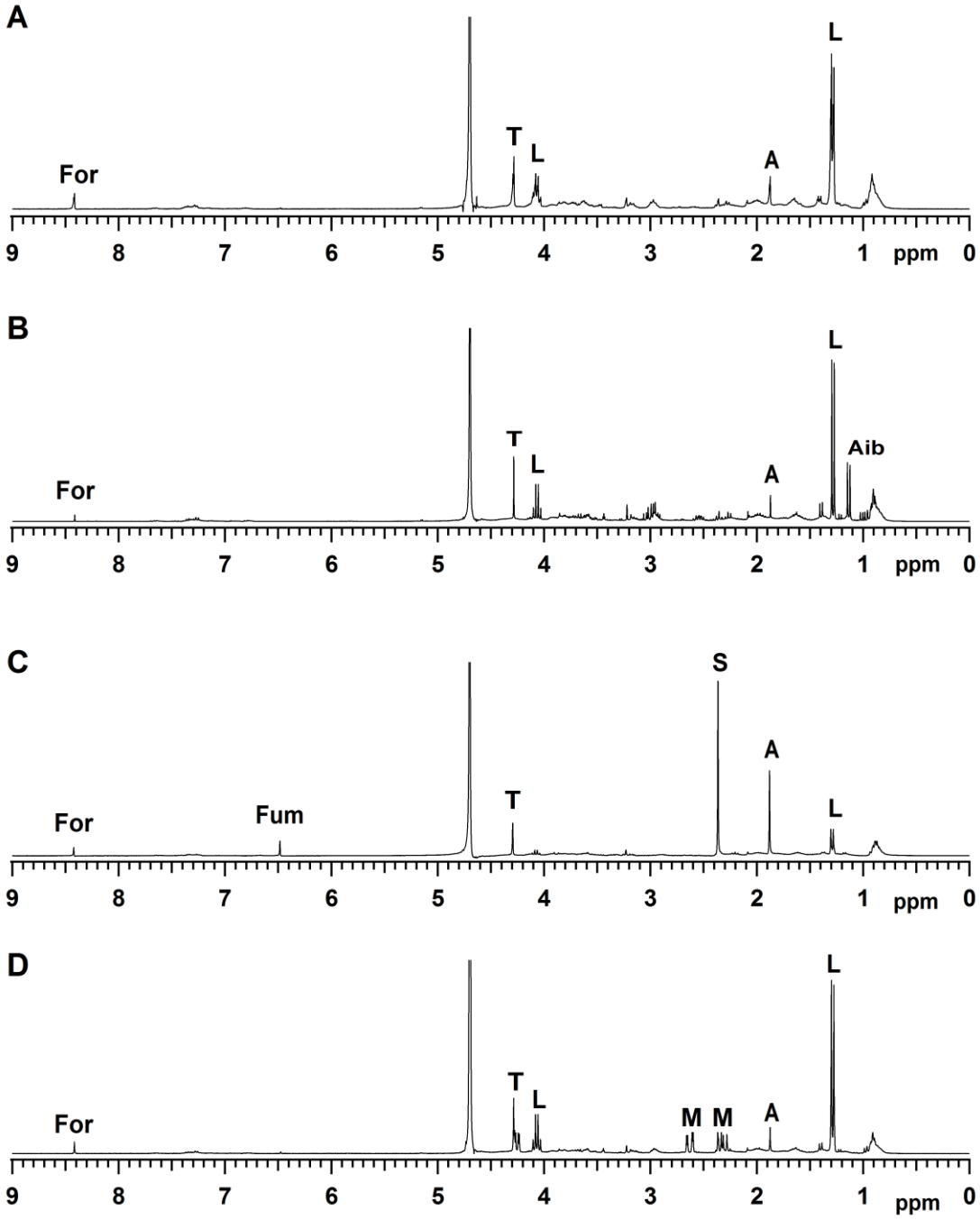


Figure-3.1: ^{1}H NMR spectra of lyophilized culture supernatants from growth cultures. A: peptone medium; B: peptone medium + 3-aminoisobutyrate (Aib); C: peptone medium + fumarate (Fum); and D: peptone medium + L-malate (M). Resonances for acetate (A) formate (For), and lactate (L) were present in each spectrum, whereas succinate (S) was found in only the fumarate-supplemented culture. Tartrate (T) was added as an internal standard.

Table-3.3: Succinate production in growth cultures supplemented with bicarbonate. Products and residual substrates in depleted peptone medium (30 mM glucose; 0–200 mM bicarbonate) at 14 h were determined by ^{1}H NMR spectroscopy.

Bicarbonate Concentration (mM)	OD_{660}	Residual Glucose (mM)	Fermentation Product (mM)		
			Acetate	Lactate	Succinate
0	1.4	nd	6	31	2
50	1.8	nd	6	37	2
100	2.4	nd	6	35	2
150	2.8	nd	9	37	3
200	2.3	nd	7	32	1

nd = not detected.

3.32 Bioconversion of fumarate to succinate

The large accumulation of succinate obtained in peptone cultures supplemented with fumarate (Table 3.2) implied a metabolic connection between fumarate and succinate. When the experiment was repeated using [2,3-$^{2}H_2$]fumarate (98% ^{2}H), the ESI(–)MS analysis (Figure 3.2) showed the accumulation of unlabelled acetate (*m*/*z* 59) and lactate (*m*/*z* 89) along with [$^{2}H_2$]succinate at *m*/*z* 119. The low intensity peak at *m*/*z* 117 indicated a high conversion of [$^{2}H_2$]fumarate to [$^{2}H_2$]succinate and little to no accumulation of unlabelled succinate. Likewise, a low intensity peak at *m/z* 115 indicated low levels of unlabelled fumarate. The labeling results help establish that succinate was produced by the reduction of exogenous fumarate with little or no synthesis of fumarate and succinate from other sources in the complex peptone medium.

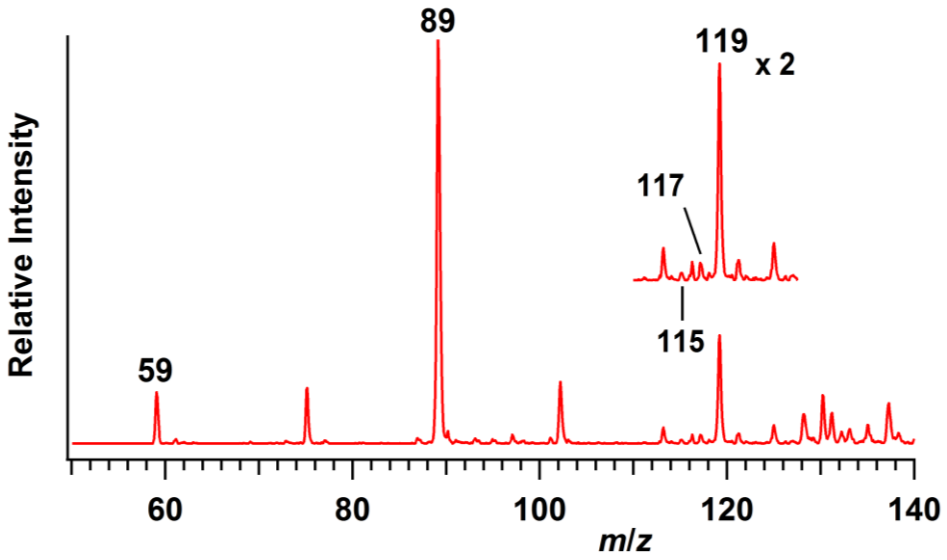


Figure-3.2: ESI(–) mass spectrum of lyophilized culture supernatant after growth of *F. varium* in the presence of [2,3-$^{2}H_2$]fumarate. The inset shows magnified intensity of the region near *m*/*z* 119.

A dependence of succinate production on substrate availability was indicated when *F. varium* was grown on peptone medium supplemented with various amounts of fumarate and glucose (Figure 3.3 and Table 3.4). Enhanced accumulations of succinate were observed as the initial concentration of fumarate was increased from 0 to 40 mM, but further increases at higher concentrations (60–100 mM) were not obtained; indicating that succinate production also depends on the availability of glucose. Incomplete utilization of fumarate was found at higher initial concentrations (≥40 mM), whereas no glucose was detected in spent culture supernatants. Thus, production of succinate was limited by low concentrations of fumarate or the supply of glucose at high concentrations of fumarate.

Table-3.4: Succinate production in growth cultures containing various combinations of glucose (10–30 mM) and fumarate (0–100 mM) in peptone medium. Residual substrates and products at 14 h were determined by ^{1}H NMR spectroscopy (plotted as Figure 3.3).

Substrate (mM)		OD_{660}	Residual Substrate (mM)		Fermentation Product (mM)		
Glucose	Fumarate		Glucose	Fumarate	Acetate	Lactate	Succinate
10	0	1.2	nd	nd	3	16	1
	20	1.8	nd	nd	11	12	14
	40	2.0	nd	7	14	10	20
	60	1.4	nd	21	18	8	19
	80	1.0	nd	35	15	8	16
	100	1.0	nd	48	18	7	18
20	0	1.5	nd	nd	7	26	4
	20	1.6	nd	nd	11	28	14
	40	1.7	nd	6	16	18	24
	60	1.8	nd	14	19	20	25
	80	1.8	nd	27	18	13	26
	100	1.8	nd	34	19	11	26
30	0	1.4	nd	nd	4	22	3
	20	2.1	nd	nd	9	26	14
	40	2.4	nd	nd	20	29	31
	60	2.2	nd	9	20	16	30
	80	2.6	nd	17	22	15	30
	100	2.2	nd	30	22	15	31

nd = not detected.

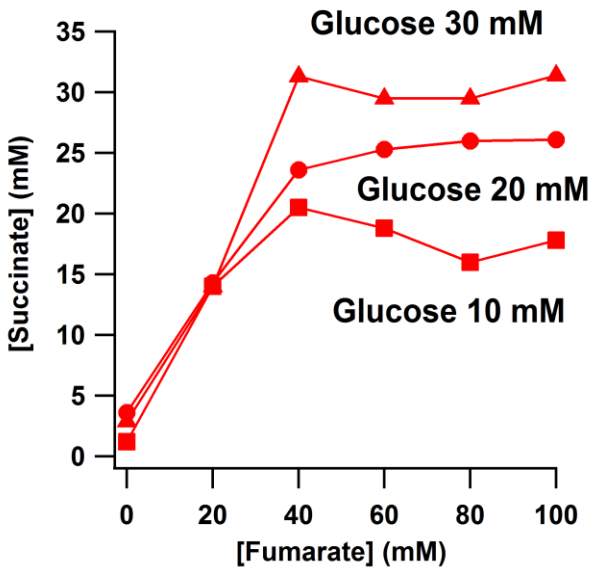


Figure-3.3: Production of succinate in *F. varium* cultures after growth for 14 h on peptone medium (10, 20 or 30 mM glucose) supplemented with varying amounts of fumarate.

The bioconversion of fumarate to succinate mediated by *F. varium* indicated that fumarate is an effective terminal electron acceptor facilitating the oxidation of reduced coenzymes formed by catabolism of glucose via the Embden–Meyerhof–Parnas (EMP) pathway.[57] The accompanying accumulations of acetate and lactate (Table 3.4) were also consistent with the metabolism of pyruvate generated by the EMP pathway. Acetate increased with increasing supplies of fumarate, while less lactate accumulated at higher concentrations of fumarate. The latter indicated that fumarate was preferred over pyruvate as a terminal electron acceptor in *F. varium* and that the coupling of glucose oxidation with fumarate reduction had potential as a viable process for succinate production under anaerobic conditions.

3.33 Succinate production by cell suspensions

F. varium cells collected from peptone growth cultures were suspended in aqueous solutions of fumarate and glucose while maintaining anaerobic conditions. Cell densities attained in the growth culture were increased in the suspensions,[91] and the results collected from a series of initial experiments established conditions for the reliable bioconversion of fumarate to succinate.

In keeping with the results collected from growth cultures (vide supra), supplies of both fumarate and glucose (source of reducing equivalents) were required to observe high conversions of fumarate to succinate (Table 3.5). Suspending cells in phosphate buffered saline (PBS) was unnecessary; effective conversions were realized using aqueous solutions of fumarate and glucose (Table 3.5). Similar efficient conversions of fumarate to succinate were attained over an initial pH range of 6.6–8.2 (Table 3.6).

Table-3.5: Effect of substrate availability on succinate production by cell suspensions. Cells collected from a growth culture (8 h, OD_{660} 2.0) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in a smaller volume of PBS or water containing glucose (0 or 30 mM) and/or fumarate (0 or 40 mM); products and residual substrates in suspension supernatants at 3 h were determined by ^{1}H NMR spectroscopy.

Suspension Conditions	Cell Density (g/L)	Residual Substrate (mM)		Product (mM)		
		Glucose	Fumarate	Acetate	Lactate	Succinate
PBS	67	nd	nd	3	3	0.9
PBS + Glucose	68	11	nd	2	28	0.7
PBS + Fumarate	68	nd	21	3	nd	5
PBS + Glucose and Fumarate	69	nd	1	10	20	31
Water + Glucose and Fumarate	68	nd	nd	12	28	32

nd = not detected.

Table-3.6: Succinate production by cell suspensions at different pH values. Cells collected from a growth culture (8 h, OD_{660} 2.7 ± 0.1) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in a smaller volume of glucose (30 mM)/fumarate (40 mM) solution (pH 6.6–8.2). Products and residual substrates in suspension supernatants at 3 h were determined by ^{1}H NMR spectroscopy.

pH	Cell Density[a] (g/L)	Residual Substrate[a] (mM)		Product[a] (mM)		
		Glucose	Fumarate	Acetate	Lactate	Succinate
6.6	81 ± 4	nd	nd	13 ± 1	27.2 ± 0.2	34 ± 4
7.0	81 ± 4	nd	nd	12 ± 2	28 ± 2	34 ± 4
7.4	81 ± 4	nd	nd	12.4 ± 0.2	27 ± 2	34 ± 2
7.8	81 ± 4	nd	nd	12.5 ± 0.4	27 ± 4	34 ± 3
8.2	81 ± 4	nd	nd	12.9 ± 0.9	26 ± 4	32 ± 2

nd = not detected.

[a] average (2 determinations) ± ½ difference between the determinations.

A comparison of cells generated from combinations of starter/growth cultures on peptone medium (± fumarate) showed that the capacity of the suspended cells to convert fumarate to succinate was increased when fumarate was included in the growth medium (Table-3.7). If fumarate was omitted from both or included in only the medium for the starter culture, the suspended cells had limited ability to produce succinate from fumarate. Furthermore, the inclusion of fumarate in the growth culture led to greater growth, and the ability of the suspended cells to produce succinate from fumarate depended on the concentration of fumarate in the growth culture (Table-3.8). Incomplete conversions of fumarate to succinate were attained by cells grown with ≤30 mM fumarate, whereas those generated by growth with ≥40 mM fumarate showed complete conversions of fumarate when suspended.

The incubation time of the growth cultures also was an important parameter. Cells collected from growth cultures incubated for 8–10 h showed greater capabilities for fumarate reduction than cells collected after longer incubations (Table-3.9).

Table-3.7: Effect of fumarate in starter and growth cultures on succinate production by cell suspensions. Starter and growth cultures were prepared on peptone medium (30 mM glucose; 0 or 40 mM fumarate). Cells collected from a growth culture (8 h) were suspended in a smaller volume of glucose (30 mM)/fumarate (40 mM) solution (pH 7.4); products and residual substrates in cell suspensions at 3 h were determined by ^{1}H NMR spectroscopy.

Starter Culture	Growth Culture		Cell Suspension					
Fumarate (mM)	Fumarate (mM)	OD_{660}[a]	Cell Density[a] (g/L)	Residual Substrate[a] (mM)		Product[a] (mM)		
				Glucose	Fumarate	Acetate	Lactate	Succinate
0	0	1.4 ± 0.2	42 ± 4	nd	24 ± 1	5 ± 1.0	40.5 ± 0.5	10 ± 3
0	40	2.2 ± 0.1	69 ± 3	nd	nd	14.5 ± 0.3	35 ± 5	37.0 ± 0.3
40	0	1.5 ± 0.1	43 ± 3	8 ± 8	31 ± 3	3 ± 1	40 ± 10	5 ± 2
40	40	2.6 ± 0.2	83 ± 3	nd	nd	15.4 ± 0.2	32 ± 4	39 ± 1

nd = not detected.

[a] average (2 determinations) ± ½ difference between the determinations.

Table 3.8: Effect of fumarate concentration in growth cultures on succinate production by cell suspensions. Cells collected from a growth culture (8 h) in peptone medium (30 mM glucose; 0–80 mM fumarate) were suspended in a smaller volume of glucose (30 mM)/fumarate (40 mM) solution (pH 7.4); products and residual substrates in suspension supernatants at 3 h were determined by ^{1}H NMR spectroscopy.

Growth Culture		Cell Suspension[a]					
Fumarate (mM)	OD_{660}	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
0	1.4 ± 0.3[b]	47 ± 2[b]	nd	21 ± 9[b]	11 ± 2[b]	36 ± 9[b]	12 ± 7[b]
10	1.9 ± 0.1	64 ± 10	nd	13 ± 11	9 ± 4	36 ± 14	17 ± 12
20	2.4 ± 0.1	76 ± 10	nd	5 ± 4	16 ± 3	26 ± 3	30 ± 2
30	2.5 ± 0.1	83 ± 7	nd	0.9 ± 0.3	16 ± 1	26 ± 4	37 ± 4
40	1.9 ± 0.1	90 ± 20	nd	nd	14.0 ± 0.2	24 ± 4	36 ± 1
60	2.2 ± 0.5	74 ± 9	nd	nd	15 ± 1	24.9 ± 0.2	33 ± 2
80	2.25 ± 0.03	78 ± 3	nd	nd	17 ± 1	26 ± 3	38.1 ± 0.5

na = not applicable; nd = not detected.

[a] average of duplicate measurements ± average of the differences between each measurement and the average.

[b] average of triplicate measurements ± average of the differences between each measurement and the average.

Table-3.9: Succinate production by suspensions of cells collected from growth cultures at various times. The cells collected from a growth culture (8–16 h) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in a smaller volume of glucose (30 mM)/fumarate (40 mM) solution; products and residual substrates in cell suspensions at 3 h were determined by ^{1}H NMR spectroscopy.

Growth Culture		Cell Suspension					
Time (h)	OD_{660}[a]	Cell Density[a] (g/L)	Residual Substrate[a] (mM)		Product[a] (mM)		
			Glucose	Fumarate	Acetate	Lactate	Succinate
8	2.6 ± 0.1	87 ± 1	nd	nd	12.9 ± 0.3	27 ± 1	37 ± 2
10	2.6 ± 0.1	85 ± 2	nd	nd	12.5 ± 0.4	29.2 ± 0.5	38 ± 7
12	2.4 ± 0.1	86 ± 3	nd	19 ± 6	8 ± 2	44 ± 3	15 ± 4
14	2.4 ± 0.1	79 ± 7	nd	22 ± 5	10.5 ± 0.7	52 ± 5	15 ± 1
16	2.3 ± 0.1	80 ± 1	nd	22 ± 4	6 ± 1	48 ± 1	11 ± 3

nd = not detected.

[a] average of duplicate measurements ± ½ difference between the measurements.

3.34 Increasing the Concentrations of Glucose and Fumarate

Various combinations of fumarate and glucose were investigated to determine the optimal amount of glucose required to support complete conversion of fumarate to succinate at high initial concentrations of fumarate to maximize the efficiency of the process (Figure-3.4 and Table-3.10). At fixed concentrations of fumarate (40 and 200 mM), succinate production depended on the amount of glucose. When glucose was supplied at approximately 60 % of the initial fumarate concentration, quantitative conversion of fumarate to succinate was obtained. At lower glucose concentrations, less succinate accumulated and residual fumarate was detected in the culture supernatant.

Thus, as seen in growth cultures (Figure 3.3), high conversion of fumarate to succinate required an adequate supply of glucose.

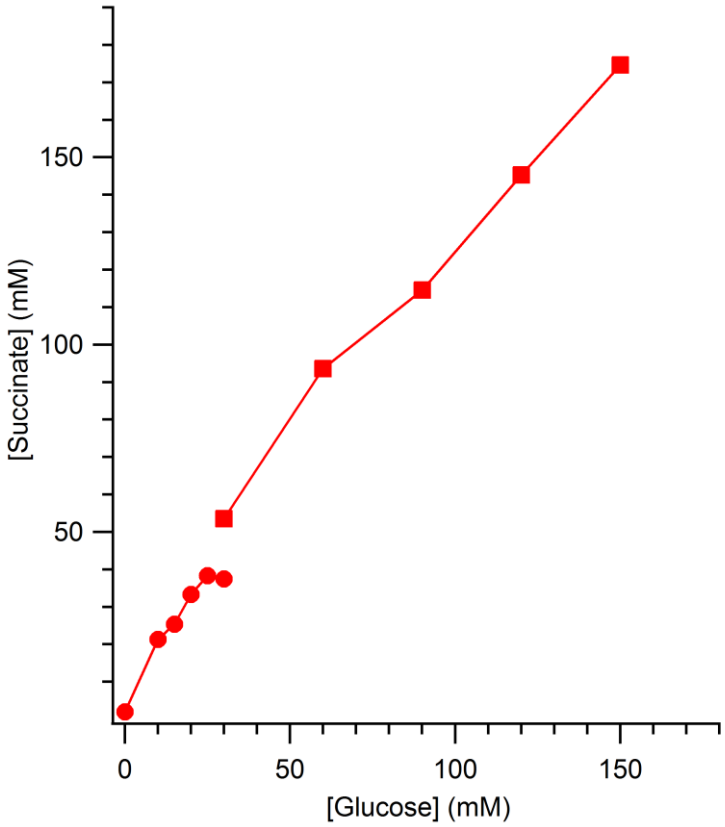


Figure-3.4: Production of succinate at 3 h by cells suspended in 40 mM (●) and 200 mM (■) fumarate at various concentrations of glucose.

Table-3.10: Effect of glucose concentration on succinate accumulation by cells suspended at different fumarate concentrations. Cells collected from growth cultures (glucose, 30 mM; fumarate, 40 mM; 8 h, OD_{660} 2.0) were suspended in glucose (10–30 mM)/fumarate (40 mM) or glucose (10–30 mM)/fumarate (200 mM) solutions (pH 7.4); residual substrates and products were determined at 3 h by ^{1}H NMR spectroscopy.

Initial Conditions			After Incubation				
Fumarate (mM)	Glucose (mM)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
40	10	67	nd	13	10	7	21
	15	66	nd	12	12	12	25
	20	68	nd	6	13	14	33
	25	67	nd	nd	13	26	38
	30[a]	67	nd	nd	13.5 ± 0.2	27.5 ± 0.2	37.5 ± 0.8
200	30	80	nd	101	17	20	54
	60	79	nd	54	31	27	94
	90	79	nd	20	39	39	115
	120	80	12	3	50	59	145
	150	81	38	2	63	95	175

nd = not detected.

[a] average of duplicate measurements ± ½ difference between the measurements.

The production of succinate as a function of time was determined by sampling cell suspensions at 30-min intervals up to 3 h (Figure-3.5, Table-3.11). At 3 h, most of the initial 200 mM fumarate had been utilized and the concentration of succinate had reached 164 mM, demonstrating that approximately 3 h of incubation was sufficient to achieve a high conversion. Except for an initial rapid uptake of fumarate, the utilization

of fumarate and the production of succinate over time were approximately linear, indicating that the cells maintained their catalytic capability over the incubation period.

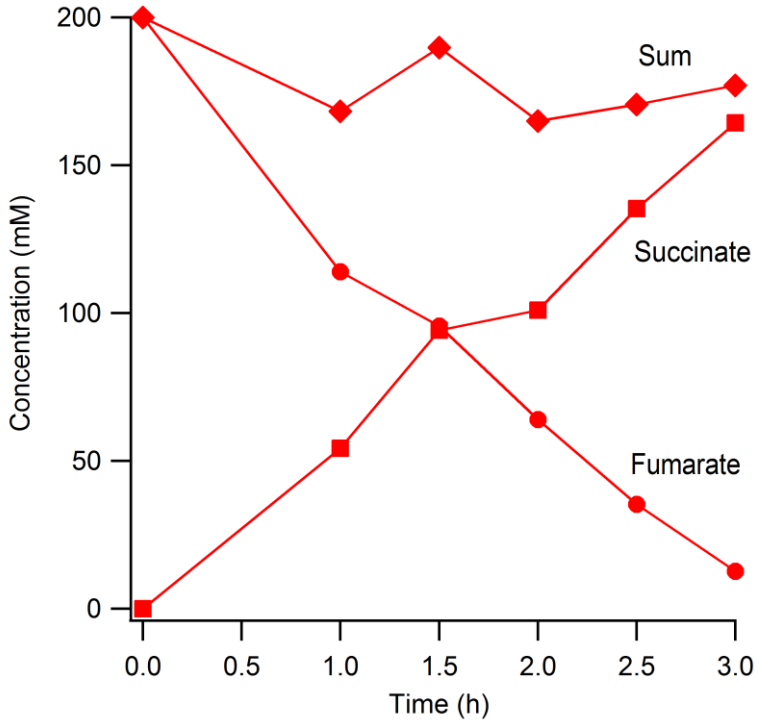


Figure-3.5: Time course of succinate production by cells suspended in 200 mM fumarate and 150 mM glucose (cell density = 67 g/L).

Table-3.11: Time course of succinate production by cell suspensions. Cells collected from a growth culture (8 h, OD_{660} 2.70) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in 10 mL of glucose (150 mM)/fumarate (200 mM) solution (pH 7.4); products and residual substrates in suspension supernatants between 1-3 h were determined by ^{1}H NMR spectroscopy.

Initial Conditions		After Incubation					
Time (h)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)	Succinate (mM/h)
1.0	67	46.7	114	15	24	54	54
1.5	67	38.7	96	22	28	94	63
2.0	67	32.0	64	38	34	101	51
2.5	67	31.3	35	53	44	135	54
3.0	67	22.3	13	60	52	164	55
							Avg: 55

In an initial experiment at 300 and 400 mM fumarate (Table-3.12), succinate accumulations were similar or less than those achieved at 200 mM fumarate with 120–150 mM glucose (Table 3.10), and the detection of large amounts of residual fumarate demonstrated partial conversion. By extending the incubation time to 6 h, higher succinate accumulations were attained at 300 and 400 mM fumarate (Table-3.13); however, the largest amount recovered was only about 70% of the initial fumarate concentration.

Table-3.12: Effect of fumarate concentration on succinate production by cells. Cells collected from growth cultures (glucose, 30 mM; fumarate, 40 mM; 8 h, OD_{660} 2.5 and 2.8) were suspended in glucose (90–210 mM)/fumarate (300 mM) or glucose (120–240 mM)/fumarate (400 mM) solutions (pH 7.4); residual substrates and products were determined at 3 h by ^{1}H NMR spectroscopy.

Initial Conditions			After 3 h Incubation				
Fumarate (mM)	Glucose (mM)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
300	90	83	19	76	51	40	133
	120	84	9	44	54	41	131
	150	83	12	38	65	52	162
	180	83	13	53	51	38	120
	210	83	37	38	50	40	124
400	120	74	nd	95	49	50	133
	150	74	nd	81	41	42	111
	180	75	nd	100	51	51	134
	210	75	nd	76	47	53	127
	240	74	nd	97	41	44	112

nd = not detected.

When the incubation at 300 mM and 400 mM fumarate was repeated using a higher cell density (Table-3.14), partial conversion of fumarate to succinate was observed after 3 h of incubation. After 6 h, the concentration of residual fumarate was negligible and the succinate accumulations were less than 70% of the initial fumarate concentration. The incubations at higher cell density showed higher succinate production at 3 h; however, the levels reached after 6 h were similar to those attained at a lower cell density (Table-3.13).

Table-3.13: Effect of fumarate concentration on succinate production by cells. Cells collected from growth cultures (glucose, 30 mM; fumarate, 40 mM; 8 h, OD_{660} 2.5 and 2.8) were suspended in glucose (90–210 mM)/fumarate (300 mM) or glucose (120–240 mM)/fumarate (400 mM) solutions (pH 7.4); residual substrates and products were determined at 6 h by ^{1}H NMR spectroscopy.

Initial Conditions			After 6 h Incubation				
Fumarate (mM)	Glucose (mM)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
300	90	83	nd	51	57	37	141
	120	84	17	30	79	49	191
	150	83	20	nd	95	76	217
	180	83	26	13	100	58	230
	210	83	31	nd	94	76	210
400	120	74	nd	65	80	68	219
	150	74	nd	52	102	77	294
	180	75	nd	55	105	81	256
	210	75	nd	38	113	91	278
	240	74	nd	61	81	63	197

nd = not detected.

Table-3.14: Effect of higher cell density on succinate production by cell suspensions. Cells collected from growth cultures (glucose, 30 mM; fumarate, 40 mM; 8 h, OD_{660} 2.4) were suspended in glucose (90–210 mM)/fumarate (300 mM) or glucose (120–240 mM)/fumarate (400 mM) solutions (pH 7.4); residual substrates and products were determined at 3 and 6 h by ^{1}H NMR spectroscopy.

Initial Conditions			After Incubation				
Fumarate (mM)	Glucose (mM)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
			After 3 h Incubation				
400	120	151	nd	55	68	46	168
	150	152	14	39	74	52	184
	180	151	39	40	95	67	225
	210	150	48	25	71	50	176
	240	152	65	34	84	59	205
			After 6 h Incubation				
400	120	151	nd	55	68	45	166
	150	152	nd	16	111	67	272
	180	151	10	1	118	80	263
	210	150	26	1	107	88	257
	240	152	51	1	111	90	269

nd = not detected.

To determine whether the build-up of succinate influenced the conversion of fumarate to succinate, it was probed by incubating *F. varium* cells with mixtures of fumarate and succinate (Table-3.15). When *F. varium* was incubated with 200 mM fumarate or a mixture of 200 mM fumarate and succinate (100 or 200 mM), no residual fumarate was detected indicating that product inhibition was not a major factor. By

contrast, partial utilization of fumarate was obtained in the parallel incubations as well as previous experiments (Table-3.12) at concentrations equal to the sum of the fumarate and succinate concentrations.

Table-3.15: Effect of succinate on succinate production by cell suspensions. Cells collected from a growth culture (8 h, OD_{660} 2.2) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in a smaller volume of glucose (150 mM)/fumarate (200–400 mM) or glucose (150 mM)/fumarate (200 mM)/succinate (0, 100 or 200 mM) solution (pH 7.4); products and residual substrates in suspension supernatants at 3 h were determined by ^{1}H NMR spectroscopy.

Initial Conditions			After Incubation				
Fumarate (mM)	Succinate (mM)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
200	0	81	33	nd	72	66	181
300	0	81	29	69	74	61	184
200	100	80	23	nd	65	56	217
400	0	81	28	172	55	34	139
200	200	80	23	nd	73	71	319

nd = not detected.

In the incubations of suspended cells, the substrate is provided as disodium fumarate, and sodium ions are a major component of the liquid medium. When *F. varium* was incubated with 200 mM fumarate supplemented with sodium chloride (Table-3.16), the residual fumarate concentration was small. The levels of succinate in the extracellular fluids, however, were less than that of the control in which no salt had been added. Thus

with salt added, the sum of succinate and residual fumarate was significantly less than the amount of fumarate added initially.

Table-3.16: Effect of NaCl on succinate production by cell suspensions. Cells collected from a growth culture (8 h, OD_{660} 2.2) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in a smaller volume of glucose (150 mM)/fumarate (200 mM)/NaCl (0–400 mM) solution (pH 7.4); products and residual substrates in suspension supernatants at 3 h were determined by ^{1}H NMR spectroscopy.

Initial Conditions		After Incubation				
NaCl (mM)	Cell Density (g/L)	Residual Glucose (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
0	76	40	10	85	40	183
100	76	34	14	69	35	148
200	76	32	10	65	37	139
300	76	22	3	67	37	148
400	76	32	4	70	41	155

This mass balance deficiency noted above for the salt experiments (Table-3.16) was also apparent in the incubations using 300 and 400 mM fumarate (Tables 3.12–3.15). Metabolism of succinate or fumarate to other products could account for the deficiency, but only acetate and lactate were detected and their levels were similar at 200, 300 and 400 mM fumarate (Tables 3.10 and 3.12). Alternatively, fumarate and/or succinate may have been retained inside the cells to maintain osmotic balance, and this would be more noticeable at the higher concentrations of fumarate or the combination of fumarate and salt.

The intracellular levels of succinate/fumarate were determined in replicate incubations of *F. varium* with 300 mM fumarate and 150 mM glucose. After 3 h of incubation, no residual fumarate was detected but the extracellular accumulation of succinate was only about 220 mM (Table-3.17). The cells collected from the incubation were suspended in 70% ethanol and the extracted material was subjected to ^{1}H NMR analysis (Table-3.17). Acetate, lactate and succinate were found in the ethanol extract. The amount of succinate extracted accounted for up to about 40 % of the deficiency noted for the extracellular concentrations. Both ethanol and acidified ethanol gave similar results. However, multiple extractions to remove substrates/productions should be performed in a future experiment to give a more quantitative result.

Table-3.17: Ethanol extraction of *F. varium* cells after incubation of cell suspensions. Cells collected from a growth culture (8 h, OD_{660} 2.6) in peptone medium (30 mM glucose; 40 mM fumarate) were suspended in a smaller volume of glucose (150 mM)/fumarate (200 mM) solution (pH 7.4) at a density of 78 g/L; products and residual substrates in suspension supernatants at 3 and 6 h were determined by ^{1}H NMR spectroscopy. The cell pellets collected at 3 and 6 h were suspended in ethanol or acidified ethanol for 1 h. The ethanol extracts were separated and evaporated. The residue was analyzed by ^{1}H NMR spectroscopy. No glucose or fumarate was detected.

Incubation Time (h)	Extracellular Concentrations (mM)			Intracellular Concentrations[a] (mM)		
	Acetate	Lactate	Succinate	Acetate	Lactate	Succinate
3	89	97	237	6[b]	8[b]	16[b]
	81	101	208	5[c]	8[c]	15[c]
6	84	98	212	12[b]	16[b]	29[b]
	76	85	225	10[c]	14[c]	29[c]

[a] Expressed as a concentration in the volume of the resuspension mixture.

[b] Extracted with ethanol.

[c] Extracted with ethanol-HCl (pH <1.0).

The significant amount of succinate remaining in the suspended cells following incubation may result from balancing the osmotic pressure when substrate/product concentrations are high. Another possibility is that ATP is needed to transfer the metabolites to outside the cell and insufficient ATP is formed to ensure the export of all products. Also, glucose may be converted into a storage polymer via an ATP-dependent process and this would account for the less than stoichiometric yields of acetate and lactate.

3.35 Other Carbon Sources:

Earlier experiments (Figure-3.3) demonstrated that the reduction of fumarate required a source of reducing equivalents supplied as NADH from the catabolism of glucose by the EMP pathway (Scheme-1.3).[57] For every mole of glucose converted to pyruvate, two moles of NADH and two moles of ATP are generated. However, other carbon sources may be more efficient substrates for fumarate reduction by contributing higher yields of NADH. For example, sorbitol, a polyol formed by reduction of glucose, yields three NADH and two ATP per mole when oxidized and degraded by the EMP pathway. Similarly, oxidation of glycerol and conversion to dihydroxyacetone phosphate, an intermediate in the EMP pathway, yields two NADH and one ATP per mole. This means that glycerol and sorbitol produce a higher ratio of NADH/ATP than glucose.

Whether sorbitol and glycerol were viable co-substrates for succinate production was assessed by testing the growth of *F. varium* on peptone medium containing one of these carbon sources (Table-3.18). Both sorbitol and glycerol supported growth and gave similar accumulations of acetate, lactate and succinate. In each case, the accumulation of succinate was stimulated by the addition of fumarate to the growth medium.

To examine the effect of carbon sources on succinate production by cell suspensions, *F. varium* was grown separately on glucose, sorbitol and glycerol and the cells were suspended in fumarate solutions containing one of the carbon sources (Table-3.19). When cells grown on a carbon source were suspended in a solution of fumarate and the same carbon source, fumarate at 40 mM was effectively converted to succinate. The cells grown on glucose had little capacity to utilize sorbitol and glycerol for the reduction of fumarate. By contrast, the cells grown on sorbitol and glycerol readily utilized glucose

and supported a high conversion of fumarate to succinate. The glycerol-generated cells were unable to utilize sorbitol to support fumarate reduction, whereas the cells generated on sorbitol had some capacity to utilize glycerol in support of fumarate reduction. Overall, these results show that the effective utilization of sorbitol and glycerol requires the cells to be primed by growth on these substrates.

Table-3.18: Succinate production in growth cultures supplemented with potential carbon sources and fumarate. Products and residual substrates in depleted peptone medium at 14 h were determined by ^{1}H NMR spectroscopy.

Carbon Source (30 mM)	Fumarate (mM)	OD_{660}	Residual Carbon Source (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
Glucose	0	1.3	Nd	nd	5	28	nd
	40	1.9	nd	nd	18	31	27
Sorbitol	0	1.7	18.0	nd	4	29	5
	40	1.1	16	nd	16	13	28
Glycerol	0	0.9	12	nd	3	3	1
	40	1.6	8	4.8	13	3	22

nd = not detected

Table-3.19: Effect of different carbon sources on succinate production by cell suspensions. Cells collected from a growth culture (8 h, OD_{660} 2.1,2.4,1.7) in peptone medium (30 mM glycerol, glucose, or sorbitol; 40 mM fumarate) were suspended in 10 mL of glycerol, glucose, or sorbitol (30 mM)/fumarate (40 mM) solution (pH 7.4); products and residual substrates in suspension supernatants at 3 h were determined by ^{1}H NMR spectroscopy.

Growth Carbon Source (30 mM)	Initial Suspension Conditions			After 3 h Incubation				
	Carbon Source (30 mM)	Fumarate (mM)	Cell Density (g/L)	Residual Carbon Source (mM)	Residual Fumarate (mM)	Acetate (mM)	Lactate (mM)	Succinate (mM)
Glucose	Glucose	0	73	9	nd	3	15	2
	Glucose	40	75	nd	nd	16	32	36
	Glucose	80	75	nd	28	19	23	50
	Sorbitol	40	74	14	35	4	nd	5
	Glycerol	40	73	29	33	5	nd	8
Sorbitol	Sorbitol	0	55	15	nd	2	4	1
	Sorbitol	40	57	6	nd	11	19	40
	Sorbitol	80	55	nd	nd	23	28	77
	Glucose	40	55	nd	nd	19	25	37
	Glycerol	40	58	8	14	9	0	26
Glycerol	Glycerol	0	64	30	nd	4	nd	4
	Glycerol	40	64	6	2	12	6	45
	Glycerol	80	63	nd	13	19	8	60
	Glucose	40	65	3	0.7	20	24	44
	Sorbitol	40	63	10	38	9	2	7

nd = not detected.

When the suspended cells were incubated with a higher fumarate concentration (80 mM) and sorbitol, quantitative conversion of fumarate to succinate was obtained. The lower conversions observed with glucose and glycerol indicated that succinate production was limited by lower yields of NADH produced by catabolism of these carbon sources (see above). Side-product formation from glucose and sorbitol were similar, but reduced levels of lactate were obtained from glycerol. The latter may be beneficial for the separation of succinate from side products in preparative scale experiments.

3.36 Most Effective Conditions for Succinate Production:

High levels of succinate production by *F. varium* were obtained only when fumarate was supplied. Unlike most of the other potential precursors tested (Table-3.1 and Table-3.2), fumarate is separated from succinate by only one metabolic step (Figure-1.4). Cells growing on complex medium supplemented with fumarate adapted and utilized fumarate as an electron acceptor whereas cells grown without exogenous fumarate had much lower capabilities to catalyze the reduction of fumarate (Table-3.4, Table-3.7, Table-3.8). Another important factor was the time in at which cells were collected from the growth culture. Cells collected at more than 10 h after inoculation were less effective at supporting succinate production from fumarate (Table-3.9). The production of succinate by cells suspensions was linear with time and most conversions were complete within 3 h (Figure-3.5, Table-3.11). Discrepancies between the initial fumarate concentrations and succinate accumulation were diminished when ethanol extracts revealed the retention of large quantities of succinate within *F. varium* cells (Table-3.17). Other carbon sources, such as glycerol and sorbitol, showed much promise

in the production of succinate as early investigations showed a production of succinate on par with glucose (Table-3.19).

Despite the large number of potential metabolic precursors connected to succinate by the citric acid cycle (Figure-1.4) and methylmalonyl-CoA (Figure-1.5), only fumarate caused succinate production to increase. This suggests that metabolism of methylmalonyl-CoA is not a major pathway in *F. varium*. Similarly, intermediates in the citric acid cycle, other than fumarate, were ineffective in stimulating succinate production, suggesting low levels of the appropriate enzymes in the citric acid cycle. The enzyme catalyzing the reduction of fumarate to succinate was likely induced in growth cultures supplemented with fumarate (Table-3.8). A candidate for the induced enzyme is fumarate reductase, which effectively catalyzes the reduction of fumarate to succinate. This reaction is in the reverse direction of the step in the citric acid cycle.

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

The hydroxyglutarate pathway and methylaspartate pathway were differentiated using isotopic labeling supplied as [$^{13}C_5$]glutamate and analysis of the butyrate products by ^{13}C NMR spectroscopy. The addition of cobalt ions to the minimal medium used to grow *F. varium* changed the metabolic pathway taken. The ^{13}C NMR coupling patterns were used to distinguish the distinct pathway taken. The results reinforce the effect of the environment on the metabolism of *F. varium*. Future work on the glutamate pathway would include further research into *F. varium* and the effects of the environment on other pathways of catabolism.

Succinate was successfully produced by *F. varium* at high conversion rates from fumarate using glucose as a carbon source. The optimal ratio of glucose to fumarate was found at lower concentrations (30 mM glucose and 40 mM fumarate) and higher concentrations (150 mM glucose and 200 mM fumarate). An upper limit was determined for the fumarate concentration (300 mM). At fumarate concentrations near the limit, succinate accumulations were consistently less than the initial fumarate concentrations. The deficiency was attributed, at least in part, to the retention of succinate within the cells. Sorbitol and glycerol were investigated as alternative carbon sources. Both were viable substrates, supporting effective conversion of fumarate to succinate. Further work to be done would involve additional ethanol extractions to obtain a more quantitative determination of succinate in the cells at high fumarate concentrations, and testing to see if sorbitol or glycerol can produce more efficient production of succinate from fumarate.

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