# A NOVEL STUDY OF THE EXTREME THERMOPHILES: RHODOTHERMUS MARINUS, SULFOLOBUS SOLFATARICUS, AND THERMOBIFIDA FUSCA

by

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LIST	OF TA	BLES	vi		
LIST	OF FI	GURES	vii		
ABST	RAC	`	.viii		
1	INTRODUCTION				
	1.1 1.2	Motivation Rhodothermus marinus, Sulfolobus solfataricus, and Thermobifida	1		
		fusca	1		
	1.3	Aim of thesis	2		
2	MA	ERIALS AND METHODS	3		
	2.1	Materials	3		
	2.2	Strain and growth conditions	4		
	2.3	Analytical methods	5		
	2.4	Mass spectrometry analysis	6		
	2.5	Metabolic network models and <sup>13</sup> C-metabolic flux analysis	7		
	2.6	Goodness-of-fit analysis	9		
3	RES	RESULTS AND DISCUSSION11			
	3.1	Growth physiology	11		
	3.2	Metabolic Model Reconstruction	13		
	3.3	<sup>13</sup> C Metabolic flux analysis	14		
		3.3.1 Metabolism of <i>Rhodothermus marinus</i>	15		
		3.3.2 Metabolism of <i>Sulfolobus solfataricus</i>	17		
		3.3.3 Metabolism of <i>Thermobifida fusca</i>	19		
4	CON	CONCLUSIONS			
	<i>A</i> 1	Conclusion	าา		
	4.2	Recommendations for future work	22		
REFE	RENC	ES	24		
Δ	דעם	A FROM S SOLFATARICUS COLUTILIZATION EXPERIMENTS	27		
B	ANN	OTATED CENTRAL CARBON METABOLISMS	29		

# TABLE OF CONTENTS

# LIST OF TABLES

Table 1. Growth characteristics of Rhodothermus marinus DSM 4252 andSulfolobus solfataricus during exponential growth on glucose......11

# LIST OF FIGURES

Figure 1. General workflow for the process of creating a flux map9
Figure 2. Growth Rate vs. Concentration data for <i>S.solfataricus</i> utilizing glucose (left) and xylose (right)
Figure 3. Progression of the growth of T.fusca beginning with cellulose generation (A), Trypsin wash (B), Water wash (C), and cellular growth (D,E,F)13
Figure 4. Metabolic flux map for <i>R.marinus</i> with glucose flux standardized to 10016
Figure 5. %13C labeling for key amino acids in the metabolism of <i>S.solfataricus</i> 18
Figure 6. A schematic describing the Weimberg and Dahms pathways used in processing xylose
Figure 7. Metabolic flux map for <i>T.fusca</i> with glucose flux standardized to 10020
Figure 8. Metabolic flux map for <i>T.fusca</i> with a cellulose flux standardized to 10021
Figure A1. LN(OD600) vs. Time for a <i>S.solfataricus</i> strain with 1:2 glucose to xylose ratio
Figure A2. LN(OD600) vs. Time for a <i>S.solfataricus</i> strain with 1:4 glucose to xylose ratio
Figure A3. LN(OD600) vs. Time for a <i>S.solfataricus</i> strain with 1:10 glucose to xylose ratio
Figure B1. Annotated metabolic map for <i>T.fusca</i> based on KEGG Pathway Database
Figure B2. Annotated metabolic map for <i>R.marinus</i> based on KEGG Pathway Database
Figure B3. Annotated metabolic map for <i>S.solfataricus</i> based on KEGG Pathway Database

#### ABSTRACT

The thermophilic organisms *Rhodothermus marinus*, *Sulfolobus solfataricus*, and *Thermobifida fusca* possess unique characteristics that make them relevant to potential industrial and biotechnological applications. *Rhodothermus marinus* grows in high salinity environments, *Sulfolobus solfataricus* grows in low pH environments, and *Thermobifida fusca* is able to utilize cellulose as its main carbon substrate. These unique abilities coupled with thermophilic organisms' innate ability to grow at high temperatures (77°C, 80°C, and 55°C for *R. marinus*, *S. solfataricus*, and *T. fusca* respectively), make these valuable candidates in the realm of biotechnology, to be utilized for tasks like production of low-boiling fuels and thermostable enzymes.

In this study, the growth physiology and metabolism of each of these organisms is investigated. Using modern tools of metabolic engineering and systems biology such as <sup>13</sup>C-metabolic flux analysis (MFA), a quantitative map of the metabolism can be generated, resulting in a greater knowledge of the organism and its by-products of metabolism.

To understand general physiology, a combination of literature review and growth experimentation was utilized. The results of the study included media, temperature, and substrate optimization, as well as revelations regarding carbon substrate sources. For instance, it was shown that *S.solfataricus* is able to grow on xylose as efficiently as it grows on glucose.

Regarding metabolism, successful characterizations of flux maps were achieved for *R. marinus* and *T. fusca*. For the *S.solfataricus* strain, initial hypotheses related to xylose processing pathways appear to have been incorrect, leaving room for further research and development of models.

## Chapter 1

## **INTRODUCTION**

## 1.1 Motivation

Within the realm of biochemical engineering applications, the use of extremely thermophilic organisms (i.e. microbes that grow optimally at temperatures of 70°C or higher) in industrial settings is becoming more prevalent as they have physical and metabolic characteristics that are advantageous to product production and recovery<sup>1</sup>. This includes aspects like high substrate uptake rates, broad substrate range, and fast doubling times<sup>1,2</sup>. However, poor understanding of cellular physiology of thermophiles presents a limitation in overall industrial applications<sup>1</sup>.

#### 1.2 Rhodothermus marinus, Sulfolobus solfataricus, and Thermobifida fusca

The three microorganisms that were studied in this thesis are *Rhodothermus marinus*, *Sulfolobus solfataricus*, and *Thermobifida fusca*. Each species was selected due to desirable growth characteristics and potential industrial applications. *Rhodothermus marinus* is a bacterium with optimal growth temperature of about 75-80°C. A key characteristic of *Rhodothermus marinus* is its ability to grow in high salinity environments. *Sulfolobus solfataricus* is a highly thermophilic archaeon with optimal growth temperature of about 80-85°C. A key characteristic of *Sulfolobus* 

<sup>&</sup>lt;sup>1</sup> Cipolla et al., 2017

<sup>&</sup>lt;sup>2</sup> Long et al., 2017

*solfataricus* is its ability to grow in low pH environments (pH<3), as well as an ability to utilize xylose as a primary carbon source. *Thermobifida fusca* is a bacterium with optimal growth of approximately 55°C. Though it is characterized as a thermophile, it does not grow at as extreme conditions as the other organisms in this study. It was primarily selected because of its intrinsic ability to process the complex carbon source, cellulose. The abilities described above for each cell line prove advantageous for potential industrial application, making each an adequate candidate for study.

## 1.3 Aim of thesis

The aim of this thesis is to apply modern metabolic engineering and systems biology techniques such as <sup>13</sup>C metabolic flux analysis, adaptive evolution, and wholegenome sequencing to elucidate key cellular characteristics for improved applications of each of the organisms. Using parallel labeling experiments, this investigation seeks to estimate intracellular metabolic fluxes of extreme thermophiles in order to provide more rational engineering targets for improving cellular properties. A more accurate model of cellular metabolism should allow for the identification of specific pathway targets for strain engineering and applications.

#### Chapter 2

#### **MATERIALS AND METHODS**

#### 2.1 Materials

Media and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Glucose tracers, [1-<sup>13</sup>C], [2-<sup>13</sup>C], [3-<sup>13</sup>C], [4-<sup>13</sup>C], [5-<sup>13</sup>C], and [6-<sup>13</sup>C]glucose (99% <sup>13</sup>C) were purchased from Cambridge Isotope Laboratories (Andover, MA). Xylose tracers, [1-<sup>13</sup>C], [2-<sup>13</sup>C], [3-<sup>13</sup>C], [4-<sup>13</sup>C], and [5-<sup>13</sup>C]xylose (99% <sup>13</sup>C) were purchased from Cambridge Isotope Laboratories (Andover, MA). Wolfe's minerals and vitamins (Cat. No. MD-TMS and Cat. No. MD-VS respectively) were purchased from ATCC (Manassas, VA). Tris solution (1 mol/L) was purchased from Cellgro (Cat. No. 46-031-CM). Yeast extract was purchased from Fisher (Cat. No. BP-1422-500, lot 068366). Glucose stock solutions (20 wt%) and yeast extract stock solution (1 wt%) were prepared in distilled water.

The base growth medium for the *Rhodothermus marinus* strain contained (per liter of medium): 0.50 g K<sub>2</sub>HPO<sub>4</sub>, 0.30 g KH<sub>2</sub>PO<sub>4</sub>, 0.50 g NH<sub>4</sub>Cl, 0.50 g NaCl, 0.20 g MgCl<sub>2</sub>.6H<sub>2</sub>O, 0.04 g CaSO<sub>4</sub>.2H<sub>2</sub>O, 40 mL of 1 M Tris, 5 mL of Wolfe's minerals, 5 mL of Wolfe's vitamins, and 0.05 g/L of yeast extract. The base growth medium for the *Sulfolobus solfataricus* strain contained (per liter of medium): 0.50 g K<sub>2</sub>HPO<sub>4</sub>, 0.30 g KH<sub>2</sub>PO<sub>4</sub>, 0.20 g MgCl<sub>2</sub>.6H<sub>2</sub>O, 0.04 g CaSO<sub>4</sub>.2H<sub>2</sub>O, 1.30 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The base growth medium for *Thermobifida fusca* contained (per liter of medium): 6.8 g Na<sub>2</sub>HPO<sub>4</sub>, 3.0 g KH<sub>2</sub>PO<sub>4</sub>, 2.0 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 g NH<sub>4</sub>Cl, 1.5 g NaCl, 0.50 g Mg SO<sub>4</sub>.7H<sub>2</sub>O, 0.015 g CaCl<sub>2</sub>.2H<sub>2</sub>O. To produce <sup>13</sup>C-labeled cellulose, the organism *Komagataeibacter xylinus* was grown in Hestrin and Schramm (HS) growth medium which contained (per liter of medium): 5.0 g Peptone, 5.0 g yeast extract, 2.7 g Na<sub>2</sub>HPO<sub>4</sub>, 1.15 g citric acid, and 20 g glucose (pH adjusted to 5.0 with HCl).

For growth of *Rhodothermus marinus*, the base growth medium was supplemented with 1% NaCl. Glucose was added as indicated in the text. For growth of *Sulfolobus solfataricus*, the base growth medium was adjusted to a pH of 3.5 using a concentrated 6 N solution of H<sub>2</sub>SO<sub>4</sub>. Glucose and xylose were added as indicated in the text. All solutions were sterilized by filtration.

## 2.2 Strain and growth conditions

Rhodothermus marinus DSM 4252 (ATCC 43812) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Sulfolobus solfataricus(ATCC 35092) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Thermobifida fusca (ATCC BAA-629) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). For parallel labeling experiments for Rhodothermus marinus and Sulfolobus solfataricus, cells from -80 °C frozen stock were first pre-grown in medium containing 2 g/L of unlabeled glucose or 2 g/L xylose. Next, 50 µL of this pre-culture was used to inoculate six culture tubes containing 10 mL of growth medium with one of six <sup>13</sup>C-glucose tracers, [1-<sup>13</sup>C], [2-<sup>13</sup>C], [3-<sup>13</sup>C], [4-<sup>13</sup>C], [5-<sup>13</sup>C], or [6-<sup>13</sup>C]glucose, or five <sup>13</sup>C-xylose tracers, [1-<sup>13</sup>C], [2-<sup>13</sup>C], [3-<sup>13</sup>C], [4-<sup>13</sup>C], and [5-<sup>13</sup>C]xylose. The optical density (OD<sub>600</sub>) of the inoculated cultures was about 0.01. Cells were then grown aerobically in custom mini-bioreactors as described previously<sup>3</sup>. Rhodothermus marinus DSM 4252 was grown at 77 °C with 2.5 g/L glucose; and Sulfolobus solfataricus was grown at 80 °C with 1.8 g/L (10mM) glucose and in some experiments, 1.8 g/L (12mM) of xylose. A high-precision multichannel peristaltic pump (Watson Marlow, Wilmington, MA) was used to control the air flow to the mini-bioreactors, which was set at 11 mL/min. Gas flow rates were monitored by a digital flow-meter (Supelco, Veri-Flow 500). Mixing in the minibioreactors was achieved through the rising gas bubbles, and a constant culture temperature was maintained by placing the tubes in a heating block (Fisher Isotemp

<sup>&</sup>lt;sup>3</sup> Swarup et al., 2014

Digital Dry-Bath 125D)<sup>4</sup>. Samples for isotopic labeling analysis were collected during mid-exponential growth phase when  $OD_{600}$  was between 0.5 and 1.0.

For parallel labeling experiments involving *Thermobifida fusca*, cells from -80 °C frozen stock were first pre-grown in medium containing unlabeled cellulose. Next, this pre-culture was used to inoculate shake flasks containing 20 mL of growth medium with one of six <sup>13</sup>C-glucose tracers incorporated into the cellulose structure,  $[1-^{13}C]$ ,  $[2-^{13}C]$ ,  $[3-^{13}C]$ ,  $[4-^{13}C]$ ,  $[5-^{13}C]$ , or  $[6-^{13}C]$ glucose. In order to incorporate the glucose tracers into cellulose, the organism *K. xylinus* (ATCC 53524) was utilized. <sup>13</sup>C-cellulose is a by-product of *K. xylinus* growth on <sup>13</sup>C-glucose. *K.xylinus* was grown aerobically in 125-mL flasks (without shaking) at 30 °C for 4 days. *T.fusca* cells were then grown aerobically in the shake flasks. *Thermobifida fusca* was grown at 55 °C with cellulose.

#### 2.3 Analytical methods

Samples were collected at multiple times during the exponential growth phase to monitor cell growth, substrate uptake and acetate production. For the *Rhodothermus marinus* and *Sulfolobus Solfataricus* strains, biomass concentration was determined by measuring the optical density at 600 nm (OD<sub>600</sub>) using a spectrophotometer (Eppendorf BioPhotometer). The OD<sub>600</sub> values were converted to cell dry weight concentrations using experimentally determined OD<sub>600</sub>-dry cell weight relationships<sup>5</sup> for *Rhodothermus marinus* DSM 4252 (1.0 OD<sub>600</sub> = 0.32 g<sub>DW</sub>/L), and *Sulfolobus solfataricus* (1.0 OD<sub>600</sub> = 0.28 g<sub>DW</sub>/L). For the *Thermobifida fusca* strain, overall biomass production was measured via dry weight following the completion of growth. OD measurements were not collected for *Thermobifida fusca* since considerable cell

<sup>&</sup>lt;sup>4</sup> Cordova and Antoniewicz, 2016

<sup>&</sup>lt;sup>5</sup> determined using the techniques described in Long et al., 2016b

clumping was observed in these cultures. . Glucose concentration was determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH), and acetate concentration was determined using an Agilent 1200 Series HPLC<sup>6</sup>.

#### 2.4 Mass spectrometry analysis

GC-MS analysis was performed on an Agilent 7890B GC system equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977A Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230°C, the MS quad temperature at 150°C, the interface temperature at 280°C, and the inlet temperature at 250°C. GC-MS analysis of *tert*-butyldimethylsilyl (TBDMS) derivatized proteinogenic amino acids was performed as described in (Antoniewicz et al., 2007a)<sup>7</sup>. Labeling of glucose in the medium was determined after aldonitrile propionate derivatization as described in (Antoniewicz et al., 2011; Sandberg et al., 2016)<sup>8</sup>. Labeling of fatty acids was determined after derivatization to fatty acid methyl esters (FAME)<sup>9</sup>. Labeling of glucose (derived from glycogen) and ribose (derived from RNA) were determined as described in (Long et al., 2016a; McConnell and Antoniewicz, 2016)<sup>10</sup>. In all cases,

- <sup>8</sup> Antoniewicz et al., 2011; Sandberg et al., 2016
- <sup>9</sup> Crown et al., 2015
- <sup>10</sup> Long et al., 2016a; McConnell and Antoniewicz, 2016

<sup>&</sup>lt;sup>6</sup> Gonzalez et al., 2017

<sup>&</sup>lt;sup>7</sup> Antoniewicz et al., 2007a

mass isotopomer distributions were obtained by integration<sup>7</sup> and corrected for natural isotope abundances<sup>11</sup>.

## 2.5 Metabolic network models and <sup>13</sup>C-metabolic flux analysis

The metabolic network models used for <sup>13</sup>C-metabolic flux analysis (<sup>13</sup>C-MFA) are provided in the appendix. For each organism, the core metabolic network model was constructed based on the pathways and reactions annotated in the KEGG and BioCyc databases<sup>12</sup>. To describe fractional labeling of metabolites, G-value parameters were included in the <sup>13</sup>C-MFA models. As described previously<sup>13</sup>, the G-value represents the fraction of a metabolite pool that is produced during the labeling experiment, while 1-G represents the fraction that is naturally labeled, i.e. from the inoculum. By default, one G-value parameter was included for each measured metabolite in each data set. The models also accounted for the dilution of intracellular labeling by incorporation of atmospheric unlabeled CO<sub>2</sub> <sup>14</sup>. Reversible reactions were modeled as separate forward and backward fluxes. Net and exchange fluxes were determined as follows:  $v_{net} = v_{f}$ - $v_b$ ;  $v_{exch} = min(v_f, v_b)$ .

All flux calculations were performed using the Metran software<sup>15</sup> which is based on the elementary metabolite units (EMU) framework<sup>16</sup>. Fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the

- <sup>13</sup> Antoniewicz et al., 2007c
- <sup>14</sup> Leighty and Antoniewicz, 2012
- <sup>15</sup> Yoo et al., 2008
- <sup>16</sup> Antoniewicz et al., 2007b

<sup>&</sup>lt;sup>11</sup> Fernandez et al., 1996

<sup>&</sup>lt;sup>12</sup> Caspi et al., 2012; Kanehisa et al., 2012; Kanehisa and Goto, 2000

experimentally measured and model predicted mass isotopomer distributions of biomass amino acids (from proteins), glucose (from glycogen), and ribose (from RNA) using non-linear least-squares regression<sup>17</sup>. Figure 1 below describes the workflow associated with the flux analysis process. For integrated analysis of parallel labeling experiments, all six data sets for each organism were fitted simultaneously to a single flux model<sup>18</sup>. Flux estimation was repeated 10 times starting with random initial values for all fluxes to find a global solution. At convergence, accurate 95% confidence intervals were computed for the estimated fluxes by evaluating the sensitivity of the minimized SSR to flux variations. Precision of estimated fluxes was determined as follows<sup>15</sup>:

Flux precision (stdev) =  $[(flux_{upper bound 95\%}) - (flux_{lower bound 95\%})] / 4$ 

<sup>&</sup>lt;sup>17</sup> Antoniewicz et al., 2006

<sup>&</sup>lt;sup>18</sup> Antoniewicz, 2015



Figure 1. General workflow for the process of creating a flux map

#### 2.6 Goodness-of-fit analysis

To determine the goodness-of-fit, the <sup>13</sup>C-MFA fitting results were subjected to a  $\chi^2$ -statistical test. Assuming that the model is correct, and data are without gross measurement errors, the minimized SSR is a stochastic variable with a  $\chi^2$ -distribution<sup>15</sup>. The number of degrees of freedom is equal to the number of fitted measurements *n* minus the number of estimated independent parameters *p*. The acceptable range of SSR values is between  $\chi^2_{\alpha/2}(n-p)$  and  $\chi^2_{1-\alpha/2}(n-p)$ , where  $\alpha$  is a certain chosen threshold value, for example 0.05 for the 95% confidence interval.

#### Chapter 3

#### **RESULTS AND DISCUSSION**

#### 3.1 Growth physiology

Growth characteristics of *Rhodothermus marinus* DSM 4252 and *Sulfolobus solfataricus* were determined in aerobic batch culture in minimal growth medium with glucose as the carbon source (Table 1). A small amount of yeast extract (0.05 g/L) was added to the media to eliminate a lag phase that was sometimes observed when subculturing cells, although the presence of the yeast extract did not impact glucose uptake rates or growth rates of the strains. *R. marinus* was grown at 77°C and Sulfolobus solfataricus grown at 80°C.

Organism	Rhodothermus	Sulfolobus
	marinus DSM 4252	solfataricus
Growth rate	$0.51 \pm 0.03 \ h^{1}$	0.081 h <sup>-1</sup>
Biomass yield	$0.38\pm0.03~g_{DW}/g$	$0.20 \ g_{\rm DW}/g$
Glucose uptake rate	$7.5 \text{ mmol/g}_{\text{DW}}/\text{h}$	$2.2 \; mmol/g_{DW}/h$

Table 1. Growth characteristics of *Rhodothermus marinus DSM 4252* and *Sulfolobus solfataricus* during exponential growth on glucose.

The specific growth rate of *R. marinus* was  $0.51 \pm 0.03$  h<sup>-1</sup> (doubling time 80 min). For *S. solfataricus*, the growth rate was slower, 0.082 h<sup>-1</sup> (doubling time 513 min) for growth on glucose. The biomass yields were  $0.38 \pm 0.03$  g<sub>DW</sub>/g and 0.20 g<sub>DW</sub>/g for *R. marinus* and *S. solfataricus* respectively.

*S. solfataricus* ' ability to grow on multiple sugars, as well as its ability to coutilize carbon sources was evaluated. The tolerance of the strain to high substrate concentrations was evaluated. For glucose, medium concentrations evaluated were 10, 20, 50, and 100mM. For growth on xylose, the evaluated concentrations were 12, 24, 60, and 120mM. The results of the growth can be observed in Figure 2 below.



Figure 2. Growth Rate vs. Concentration data for *S.solfataricus* utilizing glucose (left) and xylose (right)

The graphs above indicate that *S. solfataricus* is able to maintain growth at high concentrations, with optimal growth occurring at 10mM and 12mM for glucose and xylose respectively. The data also indicates that optimal growth on xylose occurs at a rate equal to that of glucose. In addition to concentration tolerance testing, growth experiments were carried out to evaluate the potential for co-utilization of glucose and xylose. The experiments were run with glucose to xylose ratios of 1:2, 1:4, 1:10. The results of the experiment showed growth at all ratios, with the 1:2 concentration growth rate similar to that of growth of a single carbon substrate. The resulting data and growth rates associated with these experiments can be found in the appendix.

Growth characteristics of *Thermobifida fusca* were determined via aerobic growth in shake flasks, with cellulose as the main carbon source. The cellulose used in this experiment is a by-product of the growth of the organism *K.xylinus*. Using growth medium containing 18.2 g/L of  ${}^{13}$ C-glucose, *K.xylinus* was able to be used to obtain

<sup>13</sup>C-cellulose to be used in tracer experiments. Figure 3 below shows time lapse images of *T.fusca* growth. Part A shows the cellulosic matrix with *K.xylinus* cells. B shows the culture washed with trypsin, which is used to eliminate the *K.xylinus* cells. C shows the cellulose being washed with water. D, E, and F show the growth of cells as they degrade the cellulose. As the cellulose appears to degrade, the presence of more cellular 'clumps' can be observed.



Figure 3. Progression of the growth of T.fusca beginning with cellulose generation (A), Trypsin wash (B), Water wash (C), and cellular growth (D,E,F)

## 3.2 Metabolic Model Reconstruction

Models of core metabolism were developed for the three organisms based on reactions annotated in the KEGG and BioCyc databases. Metabolic network models play a crucial role in metabolic engineering and systems biology as they are the basis for wide range of computational design and analysis approaches, including metabolic flux analysis (MFA) and flux balance analysis (FBA)<sup>19</sup>. In this study, the network models were experimentally validated using isotope tracing and <sup>13</sup>C-MFA<sup>20</sup>.

The models used for <sup>13</sup>C-MFA are provided in the appendix. The models included all major pathways of central carbon metabolism, lumped amino acids biosynthesis pathways, and a lumped biomass formation reaction. Based on current annotations, the three organisms contain many of the well-known metabolic pathways such as glycolysis, pentose phosphate pathway, TCA cycle, glyoxylate shunt, and various anaplerotic and catapletotic reactions. *R. marinus* has a complete oxidative pentose phosphate pathway (oxPPP), Additionally, *R. marinus* is believed to be missing a malic enzyme (EC 1.1.1.38). In order to experimentally validate the presence or absence of these reactions, they were all included in the <sup>13</sup>C-MFA models so that fluxes through these reactions could be estimated. For *S. solfataricus*, based on preliminary experimental data, it was suspected that the Weimberg and Dahms pathways were being used in the processing of xylose. The existence of these pathways in the model are further elaborated on later in this text.

#### 3.3 <sup>13</sup>C Metabolic flux analysis

Metabolic fluxes were determined for each tracer experiment using  $^{13}$ Cmetabolic flux analysis (MFA). COMPLETE-MFA was used to analyze combined data sets composing of either five xylose tracers or six glucose tracers. All of the data sets were fitted simultaneously to a flux model, using methods previously outlined, which resulted in a sum of squared residual values obtained via iteration. A statistically accepted fit was obtained for *R. marinus* and *T. fusca*. The model is still under evaluation, which is elaborated on below.

<sup>&</sup>lt;sup>19</sup> Long et al., 2014

<sup>&</sup>lt;sup>20</sup> Gonzalez and Antoniewicz, 2017

## 3.3.1 Metabolism of *Rhodothermus marinus*

Metabolic flux analysis was performed with the base model which included the oxidative pentose phosphate pathway, glycolysis, tricarboxylic acid (TCA) cycle, and the glyoxylate shunt. This model utilizes glucose as the primary carbon source. The results of COMPLETE-MFA are shown in Figure 4 below. The fluxes are normalized to a glucose uptake rate of 100.



Figure 4. Metabolic flux map for *R.marinus* with glucose flux standardized to 100

#### 3.3.2 Metabolism of Sulfolobus solfataricus

The model for S. solfataricus utilizes both glucose and xylose as carbon sources. Figure 5 below outlines the labeling distribution associated with the xylosebased parallel labeling experiment. From this distribution, it was hypothesized that the Weimberg and Dahms pathways were being utilized in xylose metabolism. In the Weimberg pathway, 2-keto-deoxypentonate is dehydrated to  $\alpha$ -ketoglutarate semialdehyde and oxidized to  $\alpha$ -ketoglutarate by  $\alpha$ -ketoglutarate semialdehyde dehydrogenase<sup>21</sup>. The Dahms pathway is similar to the Weimberg pathway, with the cleavage of 2-keto-3-deoxy-L-arabonate being catalyzed to pyruvate and glycolaldehyde<sup>22</sup>. The pathways are described in Figure 6 below. Metabolic flux analysis was performed with the base model, including the oxidative pentose phosphate pathway, glycolysis, tricarboxylic acid (TCA) cycle, the glyoxylate shunt, and the Weimberg and Dahms pathways. The overall sum of squared residual values obtained from <sup>13</sup>C-MFA fell outside the accepted range, which indicated that there are still issues with the proposed model for S. solfataricus metabolism. To this date, the model is still in process of re-optimization. Other xylose-based pathways are being investigated for this model.

<sup>&</sup>lt;sup>21</sup> Stephens et al. 2006

<sup>&</sup>lt;sup>22</sup> Dahms et al. 1969



Figure 5. %13C labeling for key amino acids in the metabolism of S.solfataricus



Figure 6. A schematic describing the Weimberg and Dahms pathways used in processing xylose

# 3.3.3 Metabolism of *Thermobifida fusca*

For *T. fusca*, two metabolic maps were developed, one with a normalized glucose uptake rate of 100 and one with a normalized cellulose uptake rate of 100 equivalents of glucose. Metabolic flux analysis was performed with the base model, including the oxidative pentose phosphate pathway, glycolysis, tricarboxylic acid (TCA) cycle, and the glyoxylate shunt. This model utilizes cellulose as the primary carbon source. The results of COMPLETE-MFA are shown in Figure 7 below.



Figure 7. Metabolic flux map for *T.fusca* with glucose flux standardized to 100



Figure 8. Metabolic flux map for *T.fusca* with a cellulose flux standardized to 100

#### Chapter 4

#### CONCLUSIONS

#### 4.1 Conclusion

Understanding how cells process carbon substrates and how they secrete accompanying by-products of metabolism allows for better utilization and application of these microbes in practice. This research can be in relation to and applied to a variety of applications over a range of topic areas. Improving biotechnology applications helps not only industry but has a large impact on the society that is reliant on the products of industrial efforts.

In this study, attempts were made to elucidate the physiology and metabolism of three extremely thermophilic organisms, *Rhodothermus marinus* DSM 4252, *Sulfolobus solfataricus*, and *Thermobifida fusca* using <sup>13</sup>C-flux analysis. Initial analysis helped define key aspects of growth physiology. For example, analysis revealed *Sulfolobus solfataricus*' ability to utilize xylose as efficiently as it utilizes glucose.

The analysis also allowed for the establishment of a better-defined metabolic model and flux map relating to the consumption of glucose for *Rhodothermus marinus* and the consumption of cellulose for *Thermobifida fusca*. The <sup>13</sup>C-MFA results also allowed us to resolve many model inconsistencies and fill in gaps in e.g. pathways for amino acid biosynthesis. For example, citramalate synthase was identified as playing an important role in the biosynthesis of isoleucine in *R. marinus*.

#### 4.2 **Recommendations for future work**

Based on this study, there are a variety of investigations that can be undertaken as future work. The most apparent of these is further study of *Sulfolobus solfataricus*. While this study was able to better characterize growth physiology, as well as develop an annotated metabolic map for the organism, an accurate flux map was not fully developed. Further investigation into the pathways associated with the processing of xylose is required. Should the results of further investigation result in similar model issues to that in this study (sum of squared residuals outside the accepted fit) the original experiments may need to be repeated to validate experimental data.

In addition to increased study on *S. solfataricus*, scale-up experiments can be utilized to better understand the industrial applications of the organisms. Batch or semi-batch cultures can be run using a control system, which will begin to show cell tolerance to aspects like high cell densities, microaerobic conditions, impeller speed, foaming, or general mixing issues.

Finally, the methodologies and analysis tools discussed in this study can be applied to other thermophilic organisms that can process carbon substrates like glucose, xylose, or cellulose. There are even tools and analysis methods that can investigate systems with more than one organism. Overall, further investigation into potentially useful organisms and co-cultures of multiple organisms, as well as shared environments can generate results to aid industry and benefit society.

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## Appendix A

## DATA FROM S.SOLFATARICUS CO-UTILIZATION EXPERIMENTS



Figure A1. LN(OD600) vs. Time for a *S.solfataricus* strain with 1:2 glucose to xylose ratio



Figure A2. LN(OD600) vs. Time for a *S.solfataricus* strain with 1:4 glucose to xylose ratio



Figure A3. LN(OD600) vs. Time for a *S.solfataricus* strain with 1:10 glucose to xylose ratio

## Appendix B



#### ANNOTATED CENTRAL CARBON METABOLISMS

Figure B1. Annotated metabolic map for *T.fusca* based on KEGG Pathway Database



Figure B2. Annotated metabolic map for *R.marinus* based on KEGG Pathway Database



Figure B3. Annotated metabolic map for *S.solfataricus* based on KEGG Pathway Database