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2017

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UNIVERSITY OF CALIFORNIA

Los Angeles

Synthetic ATP-independent Carbon Rearrangement Cycle
For Theoretical Conversion of Glycerol to Mevalonate

A thesis submitted in partial satisfaction of the
Requirements for the degree Master of Science
In Chemical Engineering

by

Kouki Matthew Yoshino

2017

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ABSTRACT OF THE THESIS

Synthetic ATP-independent Carbon Rearrangement Cycle For Theoretical Conversion of Glycerol to Mevalonate

by

Kouki Matthew Yoshino

Master of Science in Chemical Engineering

University of California, Los Angeles, 2017

Professor James C. Liao, Chair

In many biofuel and biochemical productions, Acetyl-CoA is an essential intermediate. In nature, routes that convert glycerol to Acetyl-CoA are not efficient in carbon conversion due to its release of CO₂ molecule. To eliminate this carbon loss step, a synthetic glycerol utilization pathway has been constructed inspired by the non-oxidative glycolysis (NOG) pathway. The new pathway, which is termed glycerol condensation cycle (GCC), is different from NOG pathway due to its ATP-independent manner. By coupling glycerol oxidation—catalyzed by glycerol dehydrogenase (GldA)—and dihydroxyacetone (DHA) condensation—catalyzed by fructose-six-phosphate aldolase (FSA)—steps with NOG pathway, glycerol compound can be converted to Acetyl-CoA without carbon loss. In addition, compared to pathways in nature, GCC pathway requires minimum number of enzymes and presents as the most thermodynamically favorable route for Acetyl-CoA production from glycerol. Enzyme amounts were adjusted using experiments from ensemble modeling, FSA glycerol inhibition, and trial and error. For

demonstrating its practicality, *in vitro* theoretical conversion of glycerol into a valuable compound mevalonate was conducted, and final carbon yield of $108.51\% \pm 25.20\%$ was achieved using purified enzymes.

This thesis of Kouki Matthew Yoshino is approved.

Yi Tang

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2017

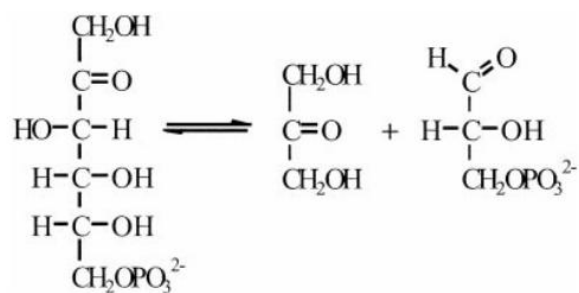
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1. Introduction

In recent years, increasing number of successful attempts have been made on genetically engineered microorganisms for higher value biochemicals or biofuels including 1,3 propanediol (Hiremath et al., 2011), lactic acid (Mazumdar et al., 2013, 2010), succinic acid (Scholten et al., 2009), 3-hydroxypropionic acid (Jung et al., 2014), fatty acid (Wu et al., 2014), ethanol (Shams-Yazdani and Gonzalez, 2008), and n-butanol (Zhou et al., 2013). However, current processes are not optimal and this limits the overall carbon conversion from glycerol to final products.

One important final product of interest is Acetyl-CoA. For producing central metabolite Acetyl-CoA from glycerol, in nature there are totally three routes. All three of these routes share a common intermediate dihydroxyacetone phosphate (DHAP). The first route of glycerol assimilation is by phosphorylation of glycerol to glycerol-3-phosphate by glycerol kinase. This step, which consumes one ATP, is followed by oxidation to DHAP with production of one reducing equivalent NAD(P)H (Figure 1A). In the second route, glycerol dehydrogenase (GldA) oxidizes glycerol to DHA. This step is then followed by phosphorylation to DHAP by consuming ATP or PEP as phosphate donor (Figure 1B). For the third route, DHA along with glyceraldehyde-3-phosphate is converted to fructose-6-phosphate (F6P) by the enzyme fructose-six-phosphate aldolase (FSA). FSA is a novel class of enzyme, where two copies of FSA recently have been characterized in *Escherichia Coli* (*fsaB* Sánchez-Moreno et al., 2012; *fsaA* Schurmann and Sprenger, 2001). This FSA is not inhibited by ethylenediaminetetraacetic acid and belongs to the class I aldolase family, which defines that no metal cofactors are required. Reaction of FSA is shown in Reaction 1.



Reaction 1

After conversion to F6P is the phosphorylation of F6P to F1,6P, which is then further broken down to G3P and DHAP by fructose-1,6-aldolase. This G3P is recycled back for next round of conversion to F6P along with DHA (Figure 1C).

Although the routes described above are all very thermodynamically favorable and energy rich (Figure 1A-C), the CO_2 release step at pyruvate dehydrogenase complex (PDHC) of EMP pathway limits one molecule of glycerol to only be converted to one molecule of Acetyl-CoA. This loss of carbon leads to significant decrease in carbon yield, which has a major impact on the overall carbon efficiency of cell growth and economy of biorefinery. To our knowledge, there is no available route to produce Acetyl-CoA from the breakdown of glycerol without the release of CO_2 .

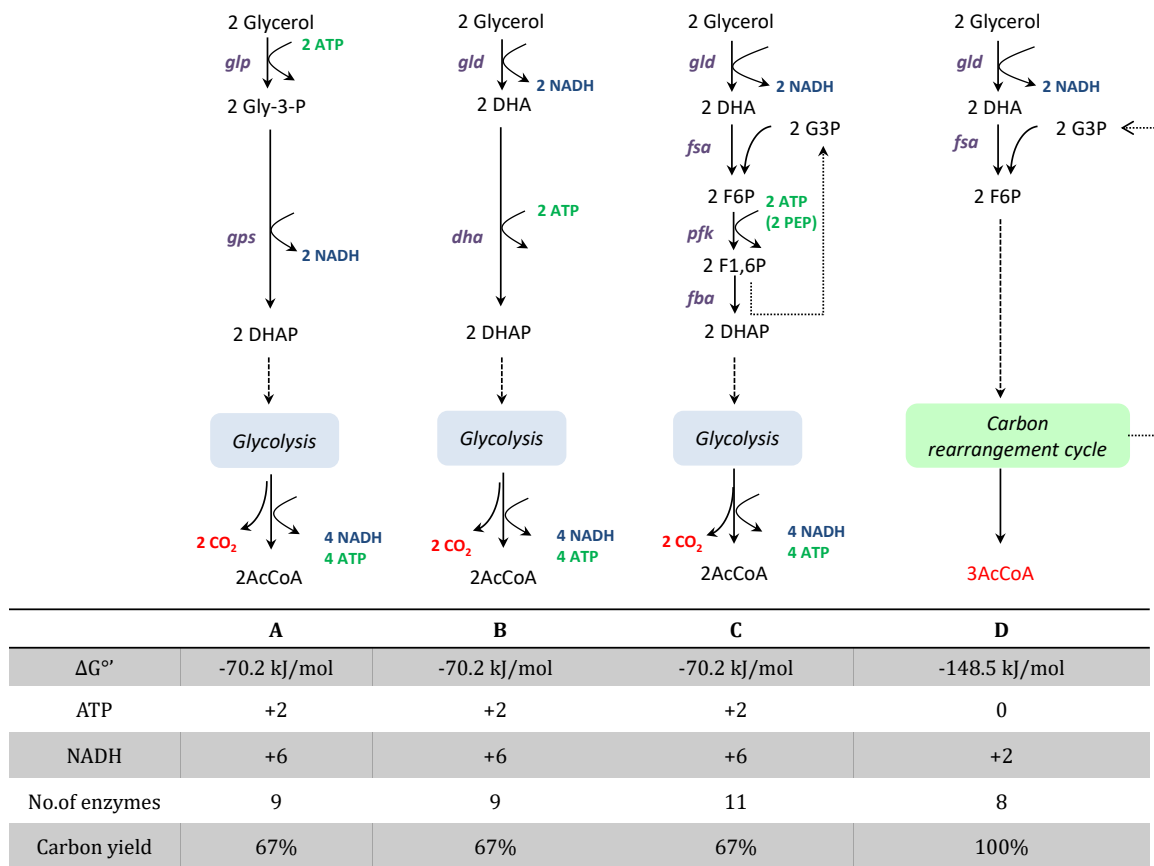


Figure 1 Schematic representation of natural and synthetic pathways for glycerol conversion to Acetyl-CoA. Natural pathway A, B, and C release two molecules of carbon dioxide through pyruvate decarboxylase reaction using EMP pathway from two molecules of glycerol uptake. Synthetic pathway D is the only pathway that converts theoretical amount of Acetyl-CoA without ATP requirement. DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, F6P: fructose-6-phosphate, F1,6P: fructose 1,6-bisphosphate, Gly-3P: glycerol 3-phosphate, G3P: glyceraldehyde 3-phosphate, AcCoA: Acetyl-CoA

Tung-Yun Wu from our lab constructed a synthetic glycerol utilization pathway to achieve theoretical carbon conversion from glycerol to Acetyl-CoA, which is then extended to produce the compound mevalonate. Mevalonate is an important precursor for isopentenyl phosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Spurgeon 1983). These two five carbon building blocks are used to make isoprenoids, which is a class of biomolecules including

vitamin K, cholesterol, heme, and all steroid hormones. In addition, IPP is a precursor to artemisinin—a drug possessing most rapid action against *Plasmodium falciparum* malaria (Martin et al., 2003; Dondorp et al., 2009).

This glycerol utilization pathway—which we term glycerol condensation cycle (GCC)—is redox-balanced, ATP-independent, and free of CO₂ release. GCC pathway is inspired by a previous work published from Professor Liao’s lab termed non-oxidative glycolysis pathway (NOG) (Bogorad et al., 2013) which converts one molecule of F6P into theoretical amount of Acetyl-phosphate (AcP). By coupling glycerol oxidation (GldA) and DHA condensation (FSA) steps with NOG pathway (Figure 1D), no carbon is released during the conversion of glycerol molecule to Acetyl-CoA. Unlike NOG pathway, however, this combined pathway is rewired in an ATP-independent manner by avoiding three enzymes: fructose-1,6-bisphosphate aldolase, fructose-1,6-bisphosphatase, and triose phosphate isomerase. In addition, inorganic phosphate is conserved in the cycle.

Compared to all natural pathways (Figure 1A-C), this synthetic pathway (Figure 1D) requires minimum number of enzymes and presents as the most thermodynamically favorable route from glycerol to Acetyl-CoA. To demonstrate the GCC pathway’s practicality, the pathway is extended from Acetyl-CoA to produce the valuable compound mevalonate by addition of three enzymes: Acetyl-CoA acetyltransferase (AtoB), HMG-CoA synthase (MvaS), and HMG-CoA reductase (MvaA). The HMG-CoA reductase converts HMG-CoA to mevalonate at the expense of 2NADH, allowing the whole pathway to be cofactor balanced. This eliminates the need for adding in further reactions to produce or consume NADH. FSA inhibition with glycerol was tested first. After finding the optimum pH of individual enzymes for the glycerol condensation

cycle, methods for stable and accurate glycerol measurement was explored. After testing ensemble modeling results to observe stability of system with variations in enzyme level, purified enzymes were used to demonstrate 100% carbon conversion of glycerol to mevalonate in an *in vitro* experiment.

2. Materials and Methods

Cloning strain *E. coli* XL-1 blue was used to propagate plasmids. *E. coli* XL-1, BL21 (DE3), or BL21 Rosetta (DE3) were used as host strain for gene expression. The recombinant *E. coli* was cultured in Luria-Bertani (LB) media supplemented with appropriate antibiotics for 16-20 hrs aerobically at 37°C. On subsequent day, 1% of overnight culture was inoculated in LB with antibiotics and cultured for 2.5-3 hrs aerobically at 37°C until OD₆₀₀ around 0.4-0.8. The culture is immediately induced with 0.5mM IPTG (isopropyl- β -D-thiogalactopyranoside) and shaken at uncontrolled room temperature (around 25°C) for 16-20hr. Cells were harvested by centrifugation at 4°C (5,500rpm) and stored in -80°C for later protein purification.

Reagents

KOD Xtreme DNA polymerases were purchased from EMD biosciences (MA, USA). Phusion Hot Start II High-Fidelity DNA polymerases were purchased from Thermo Scientific (MA, USA). DpnI enzymes were purchased from New England Biolabs (MA, USA). Glycerol dehydrogenase (*Cellulomonas sp.*) (G3512) and DL-Glyceraldehyde 3-phosphate solution (G5251) were purchased from Sigma-Aldrich (St Louis, MO).

Protein purification

Cell pellets stored in -80°C were resuspended in 30mL of 50mM GlyGly buffer (pH7.5) and were disrupted using sonicator. Cell debris was removed by centrifugation at 18,000rpm for 1hr

and supernatant was used for protein purification. The supernatant was then subject to filtration. The purification was conducted using Profinia Affinity Chromatography Protein Purification System (Bio-Rad). In cases of purifying multiple enzymes in a single run, the purification was conducted at room temperature using P1 column from Zymo Research (CA, USA) with Ni-NTA resin from Thermo Scientific (MA, USA). All the buffers are glycylglycine-based buffers. Protein concentration was measured by Coomassie Plus Assay (Thermo Scientific) at OD₅₉₅. All the enzymes except *fpk* were stored in 4X protein stabilization cocktail (Thermo Scientific) in either 4°C or -20°C. Enzyme *fpk* was stored in -20°C with 20% glycerol.

Enzyme assay for FSA characterization

Standard FSA activity assays were carried out in a 200 µL assay mixture containing 50 mM glycylglycine buffer (pH 8.5), 0.1 µg of FSA, 5 mM of Mg²⁺, 3 mM NADP⁺, at 37 °C. For pH assays, buffers pH 6 (2-(*N*-morpholino)ethanesulfonic acid), pH 7 (potassium phosphate), pH 8.5 (glycylglycine), pH 9.5 (sodium bicarbonate)

Glycerol to Mevalonate production assay

The assay pH was chosen at pH 8.5 using Gly-Gly buffer as a compromised condition between optimum activity between GldA and F/Xpk. The reaction mix contained 50mM Gly-Gly buffer, 3mM NAD⁺, 1mM K₂HPO₄, 2.2mM DL-G3P, 40mM Sodium formate, 0.2mM Coenzyme A, 5mM MgCl₂, 5mM KCl, 1mM thiamine pyrophosphate, and 100-200mM of glycerol. The amount of enzymes per 200ul reaction were as follows: GldA 2U (5µg), FsaA(A129S) 77µg,

TalB 320µg, TktA 80µg, Rpi 57µg, Rpe 19.3µg, Pta 30µg, AtoB 168µg, MvaS 120µg, MvaA 127µg. The reaction mix was prepared without Fpk then waited for 10 minutes. Next, 85µg of Fpk was added to initiate the whole reaction. All reactions were performed in 1.5ml microcentrifuge tube at 30°C in a rotary shaker at 550rpm. At each time point, 100ul of sample was taken and the reaction was quenched with 10% of 0.5M H₂SO₄ by dropping the reaction pH. Samples were then incubated for 2hrs at 30°C in a rotary shaker at 550rpm to stabilize sample.

Glycerol, DHA, and Mevalonate Derivatization

Glycerol and DHA Derivatization: 20 µL of sample was diluted with 280 µL of water. The diluted sample was filtered using Whatman mini-uniprep filter vials (GE Healthcare Life Sciences). Sample was analyzed using High Pressure Liquid Chromatography.

Mevalonate Derivatization: To 20µL of sample, 1µL of 4M HCl was added. The solution was incubated at 45°C for one hour. 10µL of 0.01 mg/mL hexadecanol was added as internal standard. 200µL of ethyl acetate is added as extracting solvent (should form two distinct phases). Supernatant was analyzed by Gas Chromatography Mass Spectrophotometer.

3. Result

3.1 Pathway design for mevalonate production

The pathway was extended from Acetyl-CoA to mevalonate to demonstrate the synthetic pathway's feasibility. Two glycerol molecules can be rearranged into three Acetyl-CoA molecules and yielded two reducing equivalents NAD(P)H, which can be used to produce one (*R*)-mevalonate molecule.

NOG rearranges the G6P produced from condensation of DHA and G3P and produces theoretical amount acetyl-phosphate (AcP) by the key enzyme phosphoketolase (F/Xpk). This promiscuous enzyme either catalyzes the irreversible cleavage of F6P (Fpk) or X5P (Xylulose-5-phosphate) (Xpk) into AcP and their corresponding sugar phosphates erythrose 4-phosphate (E4P) and G3P, respectively. In Figure 2A and B, two variants of carbon rearrangement pathways are shown, both of which results in same net conversion. Xpk form is assumedly dominant due to its low K_m towards to X5P among characterized phosphoketolase. Theoretically, every 2 glycerol can be converted to 3 Acetyl-CoA, which is 33% carbon yield improvement compared to use of EMP pathway. Conjointly, the irreversible cleavage step of phosphoketolase creates a driving force that represents an overall reactions to be the most favorable route of Acetyl-CoA production from glycerol (Figure 1D). List of enzymes and its reactions are listed in Table 1.

Our synthetic pathway converts 2 glycerol molecules into 3 Acetyl-CoA molecules and 2 NADH are generated (Figure 2). The 3 Acetyl-CoA molecules can then be converted into 1

molecule of mevalonate with consumption of 2 NADH. This allows the pathway to become cofactor balanced and still remains ATP-independent.

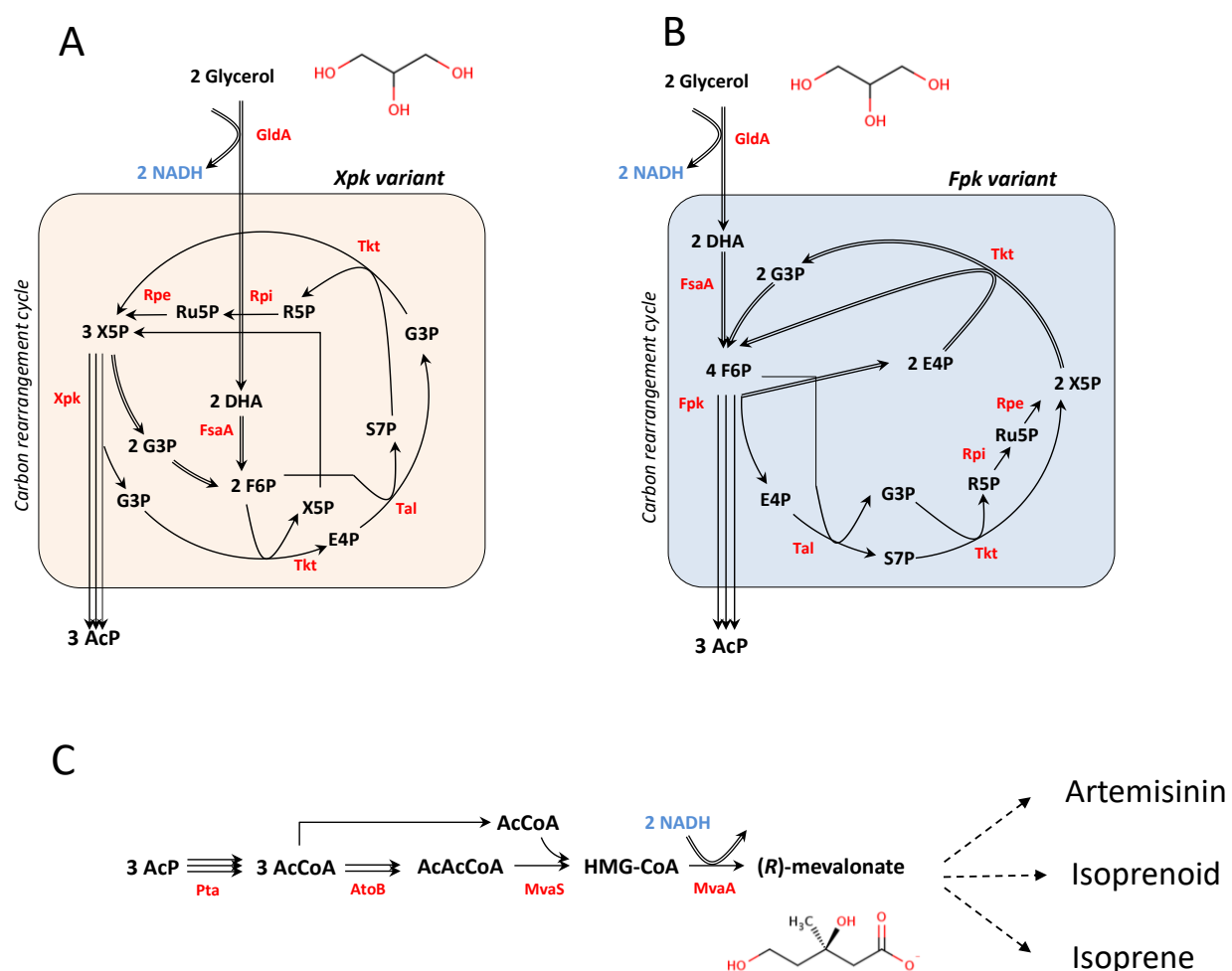


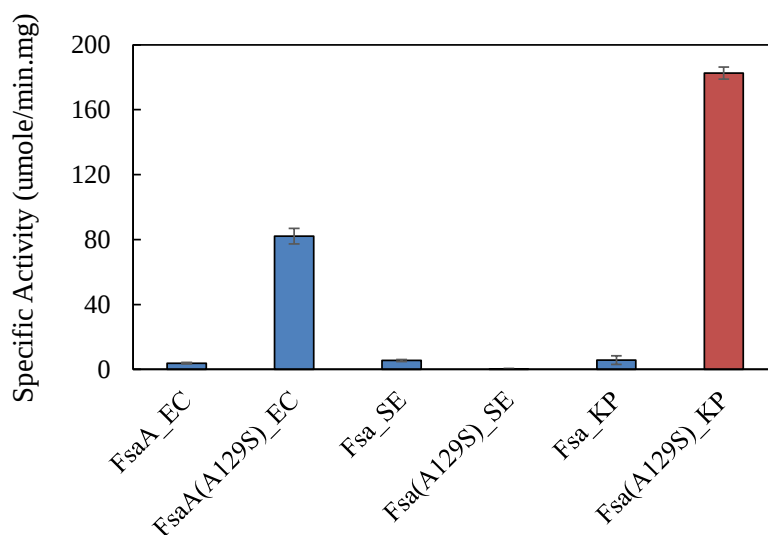
Figure 2 (A) Xpk and (B) Fpk variants of carbon rearrangement cycles for glycerol utilization pathways. Both rearrangement cycles can achieve 100% carbon yield. (C) AcP conversion to (R)-mevalonate. AcP: acetyl-phosphate, AcCoA: Acetyl-CoA, AcAcCoA: acetoacetyl-CoA, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.

Table 1. Enzymatic reactions of ATP-independent Glycerol to mevalonate pathway

Gene	Enzyme	Reaction	EC number
GldA	glycerol dehydrogenase	Gly + NAD ⁺ $\rightleftharpoons$ DHA + NADH	1.1.1.6
FsaA (A129S)	fructose 6-phosphate aldolase I	F6P $\rightleftharpoons$ DHA + G3P	4.1.2. -
TalB	Transaldolase	F6P+E4P $\rightleftharpoons$ G3P+S7P	2.2.1.2
TktA	transketolase I	G3P+S7P $\rightleftharpoons$ R5P+X5P	2.2.1.1
TktA	transketolase I	E4P+X5P $\rightleftharpoons$ F6P+G3P	2.2.1.1
Rpe	ribulose-5-phosphate 3-epimerase	Ru5P $\rightleftharpoons$ X5P	5.1.3.1
Rpi	ribose-5-phosphate isomerase A	R5P $\rightleftharpoons$ Ru5P	5.3.1.6
Fpk	Phosphoketolase	F6P + Pi $\rightarrow$ AcP + E4P	4.1.2.22
Xpk	Phosphoketolase	X5P + Pi $\rightarrow$ AcP + G3P	4.1.2.9
Pta	phosphate acetyltransferase	AcP + CoA $\rightleftharpoons$ AcCoA + Pi	2.3.1.8
AtoB	Acetyl-CoA acetyltransferase	2 AcCoA $\rightleftharpoons$ AcAcCoA + CoA	2.3.1.9
MvaS	HMG-CoA synthase	AcAcCoA + AcCoA $\rightarrow$ HMG-CoA	2.3.3.10
MvaA	HMG-CoA reductase	HMG-CoA + 2NADH $\rightarrow$ Mevalonate	1.1.1.88

Gly: glycerol, F6P: fructose 6-phosphate, G3P: glyceraldehyde 3-phosphate, DHA: dihydroxyacetone, E4P: erythrose 4-phosphate, S7P: sedoheptulose-7-phosphate, R5P: ribose 5-phosphate, Ru5P: ribulose 5-phosphate, X5P: xylulose 5-phosphate, AcP: acetyl-phosphate, AcCoA: Acetyl-CoA, CoA: coenzyme A. AcAcCoA: acetoacetyl-CoA, HMG-CoA: hydroxymethylglutaryl-CoA

One of the key enzymes in the GCC pathway is FsaA, which Schurmann and Sprenger reported to be the first enzyme found from any organism to be catalyzing the aldol cleavage of fructose 6-phosphate (Schurmann and Sprenger, 2001). Wild –type FsaA has high K_m towards DHA (32 ± 2 mM) and low catalytic efficiency to both substrates DHA and G3P. A mutant version of FsaA(A129S) with 17-fold improved catalytic efficiency to DHA and 2-fold to G3P was cloned and used from the published work (Castillo et al., 2010). Results of K_m to DHA and G3P, along with the V_{max} of different FSA mutants are summarized in Figure 3.



	Km (DHA)	Km (G3P)	Vmax (U/mg)
FsaA_EC	54.5 ± 5.4	0.18 ± 0.04	16.1 ± 0.7
FsaA(A129S)_EC	12.3 ± 2.6	0.46 ± 0.04	111.4 ± 8.5
Fsa_KP	15.1 ± 0.4	0.19 ± 0.04	8.8 ± 0.4
Fsa(A129S)_KP	9.8 ± 1	0.92 ± 0.07	216.2 ± 6.1

Figure 3 Characterization of fructose-6-phosphate aldolase and effect of site-directed mutagenesis on A129S.

One major problem with wild-type FsaA is that the activity is inhibited by glycerol (Schurmann and Sprenger, 2001). To test the activity of mutated FsaA (A129S) with presence of glycerol, Fsa(A129S) was incubated with 25mM glycerol for 1 hour. Comparing the FSA before and after glycerol incubation, the activity dropped by half from 180 umole/min.mg to 90.11 umole/min.mg respectively. Although the activity dropped by 50%, the FSA activity was still strong enough for the mevalonate production and FSA enzyme amount was increased to account for the inhibition. As a precaution to minimize glycerol inhibition, FSA was the second to last

enzyme added in the mixture for *in vitro* production (last enzyme added is Fpk to initiate pathway).

3.2 Finding optimum pH of pathway enzymes

One of most important factor in constructing a synthetic pathway *in vitro* is to provide a working environment for the synergy of all enzymes to exhibit moderate activity. For instance, due to thermodynamic barrier of glycerol oxidation reaction, basic pH is required to drive this reaction forward and thus GldA optimum activity occurs at pH9.5 (Figure 4). On the other hand, optimum activity of FXpk is at neutral pH (pH 7). To investigate the optimal pH conditions that could be used for our *in vitro* production environment, four pH conditions on individual enzymes were tested. Assay results indicated that there are 10 different enzymes showing optimum activity at pH7 and 8.5 conditions. One key enzyme, GldA, worked optimally at pH9.5. However, four carbon rearrangement enzymes (TalB, TktA, Rpe, Rpi) with the addition of FsaA(A129S) preferred at condition of pH8.5. In order to drive thermodynamically unfavorable glycerol oxidation reaction as well as promote DHA condensation to carbon rearrangement cycle, the condition pH8.5 was chosen for the *in vitro* analysis in this study.

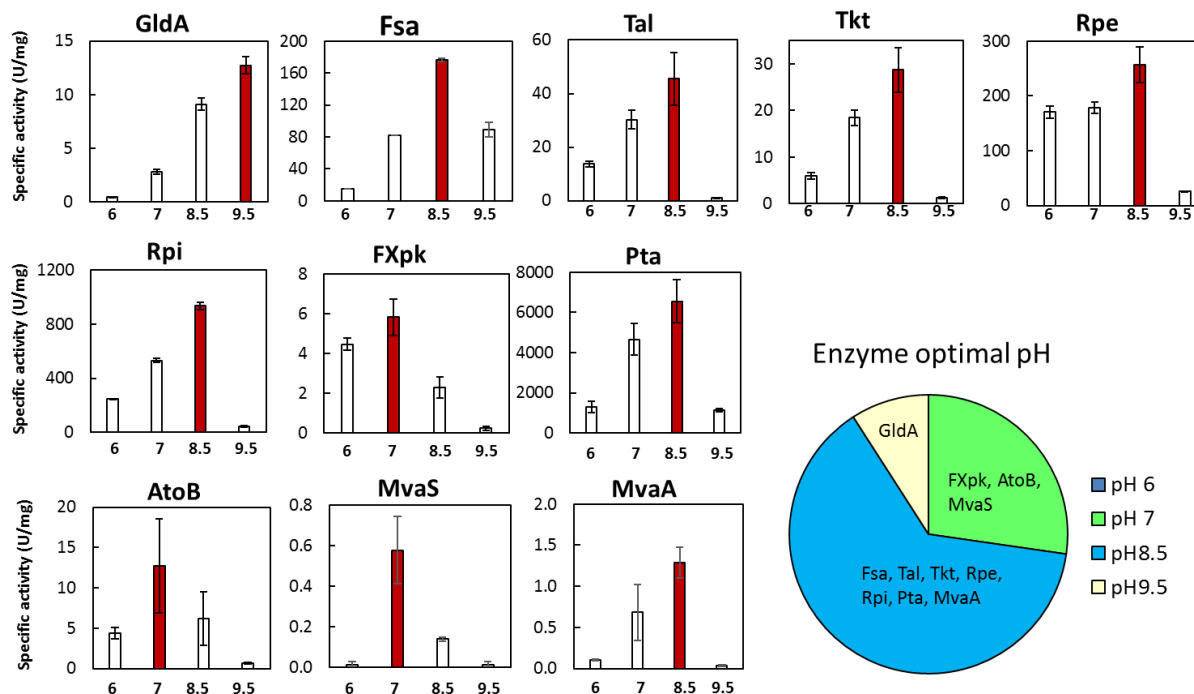


Figure 4 Displaying optimum pH of individual enzymes for glycerol condensation cycle. Key carbon rearrangement enzymes are optimum at pH 8.5, leading to deciding the pH for *in vitro* production environment as pH 8.5.

3.3 Finding optimum glycerol sample preparation method

Before testing the optimal enzyme levels for our *in vitro* production, optimal sample preparation methods were investigated. Reason for this is a topic discussed also later in the thesis about sensitivity of the sample due to glycerol measurement errors. Mevalonate measurement has been very accurate with very little error. For glycerol measurements, the results were non-reproducible in the initial phases of testing. Three main changes were made to improve on the accuracy of sample glycerol measurement.

The first change was changing concentration of acid used. Initially, 0.25M sulfuric acid was utilized to quench the reaction. We tested 0.25M sulfuric acid, 1M sulfuric acid, and 1M

HCl to observe the deviations in results of triplicate data. As seen from Figure 5, we see that 1M sulfuric acid yielded the smallest standard deviation based on sets of triplicate data.

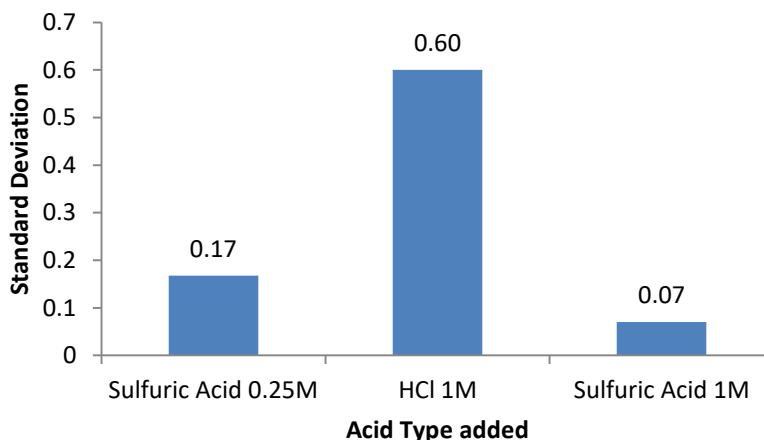


Figure 5 Testing standard deviation of glycerol measurements when quenched with different acids. 1M sulfuric acid yielded smallest standard deviation.

To avoid contamination in HPLC machines, all samples are subject to filtration using Whatman filter vials before glycerol measurement. One flaw of these filter vials is the need for at least 200 μ L of solution before the sample passes through the vial filter (Figure 6A). This leads to the need of diluting samples since we cannot use 200 μ L of sample for each time point (0, 2, 4, 6, 20, and 24 hour) with triplicates for each time point. Dilution factors tested in this experiment are: 20 μ L sample + 280 μ L water, 20 μ L sample + 580 μ L water, 30 μ L sample + 770 μ L water, and 50 μ L sample + 950 μ L water. From the results displayed in Figure 6B, we observed that the 20 μ L sample + 280 μ L water dilution had the least standard deviation out of all the dilution factors.

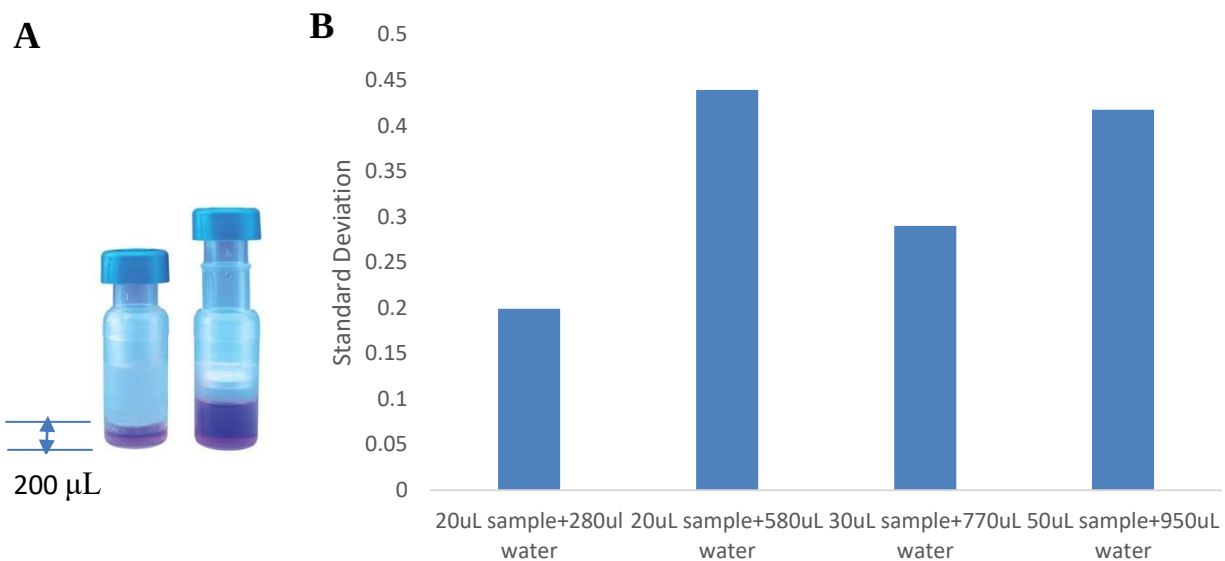


Figure 6 A) Picture of Whatman filter vials used in experiment, and arrow represents volume of 150 µL needed before sample passes through filter. **B)** Represents standard deviation with respect to triplicate data of glycerol measurements for different dilutions of sample.

Last change made was altering machine and method used for measuring glycerol.

Previously, glycerol measurements were conducted using gas chromatography mass spectrophotometer (GC-MS). For this measurement, derivatization was conducted by taking 20µL of sample and adding with 1µL of 4M HCl and 20µL of 1-imidazole. Next, 100µL of 100% acetic anhydride was added and waited for five minutes. 10µL of 10X hexadecanol was then added as internal standard, followed by addition of 200µL 100% ethyl acetate as extraction solvent. In comparison, HPLC method required only diluting the sample and subjecting the diluent to Whatman filter vial. There is far less chance for human error, and the results with GC-MS were very inconsistent. Measurement with another machine named glycerol analyzer was also attempted, but problems with calibration of glycerol standards created major measurement errors. As seen from Figure 7A, glycerol measurement decreases in HPLC, while glycerol values increase in GC-MS measurement. In this run, mevalonic acid production of 0.65 g/L was measured at 6 hours. With production of mevalonate, the data does not make sense for

glycerol to increase since no other carbon sources are supplied. Thus, HPLC became the instrument of choice for glycerol measurement.

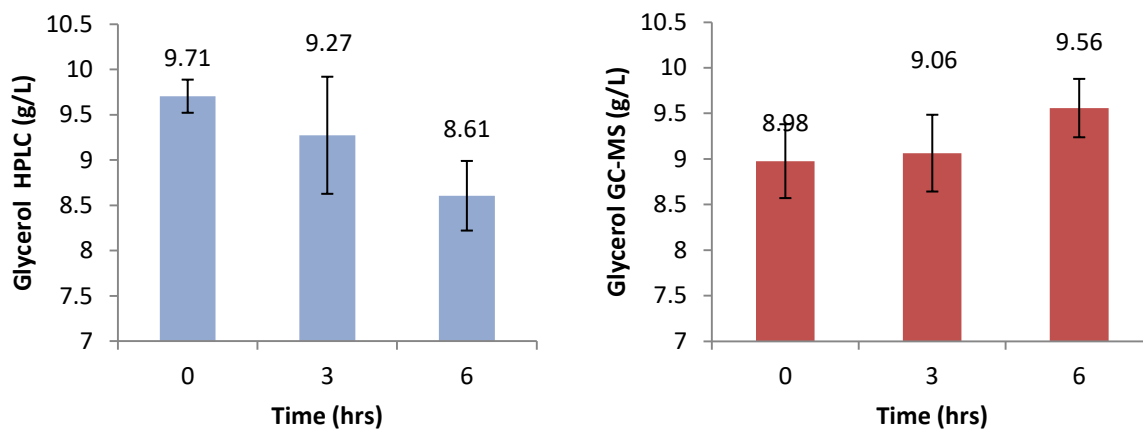


Figure 7 A) Measurement using HPLC. Glycerol value decreases and demonstrates consumption. B) Measurement using GC-MS. Despite production of mevalonate during this experiment, results show production of glycerol, which is not feasible.

3.4 Validating Ensemble Modeling results with *in vitro* experiments

To find the optimal level of enzymes needed to conduct an *in vitro* production of mevalonate, we first had our lab member create an ensemble modeling system to simulate the effects of various enzyme levels on the pathway. The effects of stability of the pathway with perturbations in enzyme activity is simulated and represented in Figure 8.

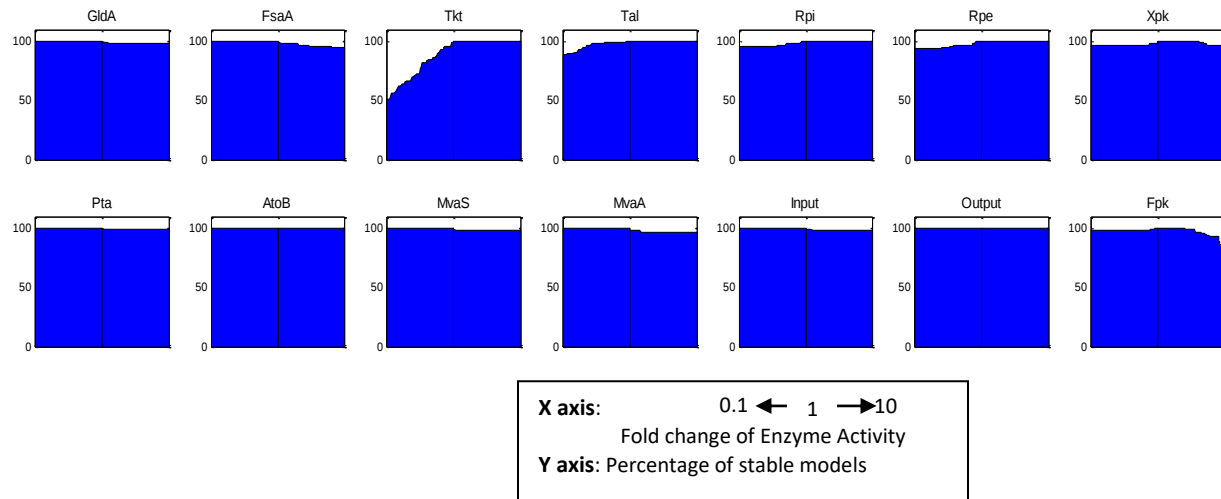


Figure 8 Represents the change of stability of GCC pathway with change in enzyme activities. Tkt can be seen here to be crucial for stability of the pathway, while too much Fpk activity can be detrimental to the stability of the pathway.

As seen from the figure above, enzymes Tkt and Fpk create perturbations in the stability of GCC pathway. When Tkt enzyme activity is low, X5P generation in the Xpk variant of GCC cycle is slowed down and causes the overall pathway to slow down. The Fpk variant of GCC cycle is also affected due to the low generation of G3P needed for the production of F6P. As for Fpk, a kinetic trap occurs due to too high activity of FPK draining the available F6P. This creates problems because the GCC cannot produce the G3P needed to regenerate F6P, thus compromising the activity of GCC. To test the ensemble modeling results, we tested Fpk amounts of 0.125 $\mu\text{g}/\mu\text{L}$, 0.425 $\mu\text{g}/\mu\text{L}$, 0.6 $\mu\text{g}/\mu\text{L}$, 1 $\mu\text{g}/\mu\text{L}$, 1.5 $\mu\text{g}/\mu\text{L}$, and Tkt amounts of 0.04 $\mu\text{g}/\mu\text{L}$, 0.2 $\mu\text{g}/\mu\text{L}$, 0.4 $\mu\text{g}/\mu\text{L}$, 0.8 $\mu\text{g}/\mu\text{L}$, 1.6 $\mu\text{g}/\mu\text{L}$. The results of mevalonate production with changes in these two enzyme amounts are represented in Figure 6.

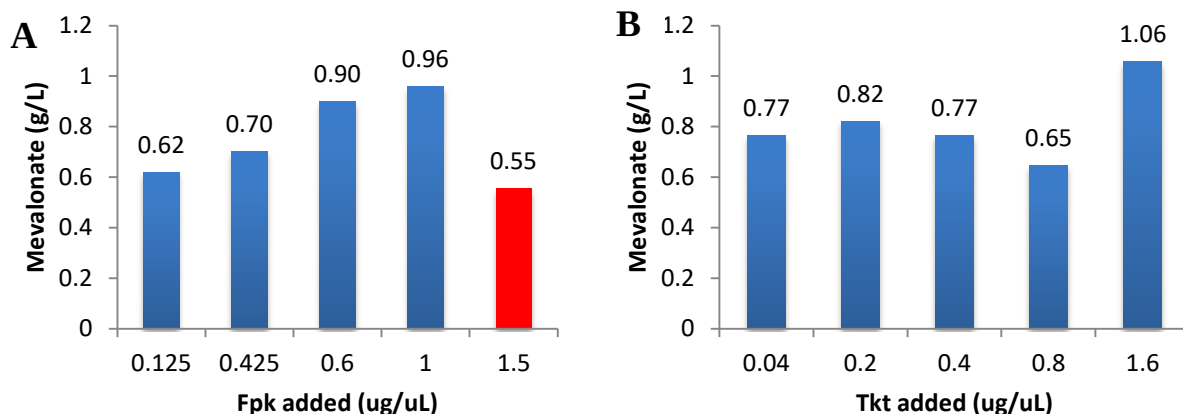


Figure 9 A) Mevalonate production with change in Fpk. When Fpk amount is highest at 1.5 $\mu\text{g}/\mu\text{L}$, the mevalonate production drops by roughly 42%. **B)** Mevalonate production with change in Tkt. With changes in Tkt enzyme variations, no visible significant changes in mevalonate production seen corresponding to ensemble modeling results.

As seen from Figure 9A above, mevalonate production at 1.5 $\mu\text{g}/\mu\text{L}$ of Fpk drops by roughly 42% compared to that of 1 $\mu\text{g}/\mu\text{L}$. Fpk is stored using glycerol, so increasing Fpk amount increases the glycerol starting amount and hence increase the mevalonate production. However, since mevalonate production drops, this demonstrates that the kinetic trap limits production of mevalonate with increase of Fpk. The trend of ensemble modeling results matches with the trend seen here in the mevalonate production when Fpk activity is greatly increased.

Meanwhile, mevalonate production with changes to Tkt did not vary significantly (Figure 9B). Although the mevalonate production increased when 1.6 $\mu\text{g}/\mu\text{L}$ of Tkt was added, the change was not significant. We can state the change is not significant because addition of more enzymes equates to more glycerol in system—more glycerol leads to greater mevalonate production—since all the enzymes are stored in protein cocktail containing 20% glycerol. The trend for Tkt did not match with ensemble modeling simulation, and believed that the Tkt activity was still high even with 8 μg .

3.5 Demonstration of mevalonate production

Lastly, we conducted long term experiments to demonstrate the conversion of glycerol to mevalonate. To first find which enzyme was the limiting step or was needed in larger quantity, each enzyme in the GCC pathway was doubled and tested for *in vitro* production test of mevalonate. The results are represented below in Figure 10.

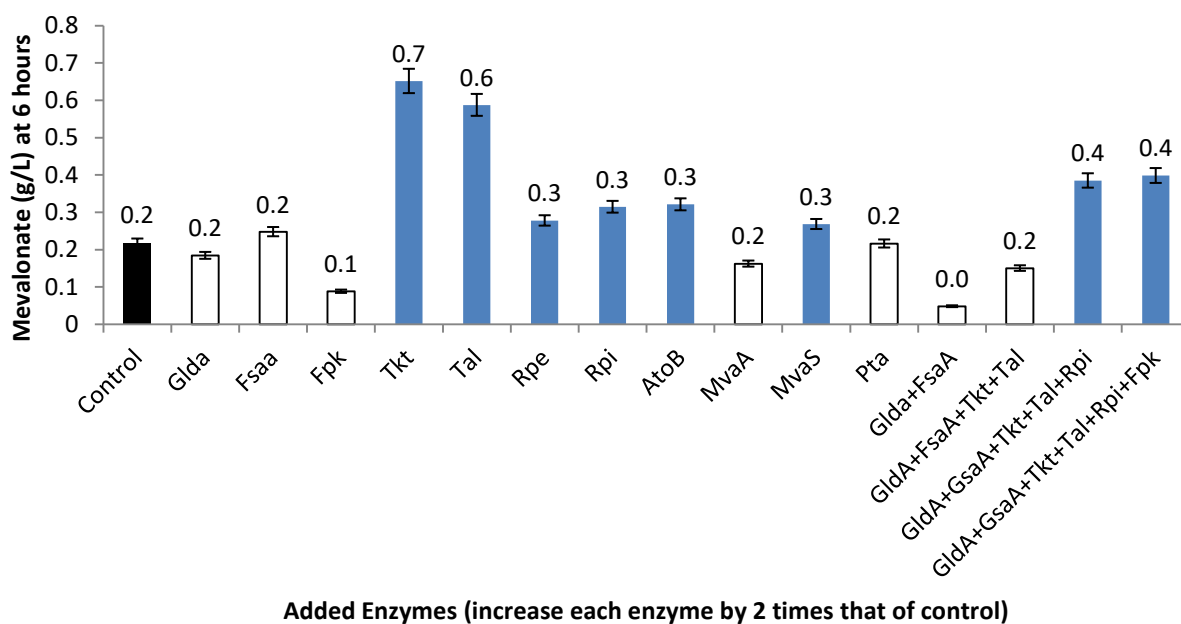


Figure 10 Changes in mevalonate production at 6 hour time point by varying enzyme amount. Last four runs are combinations of adding 2X of the enzymes in the set. All white bars represent no difference or smaller production level compared to control. All blue bars represent increased mevalonate production level compared to control. Error bars indicated standard deviation (s.d.).

After doubling Tal, Tkt, Rpi, Rpe, AtoB, and MvaS, another round of increasing different enzymes were conducted. Results for the second-round adjustment are represented in Figure 11.

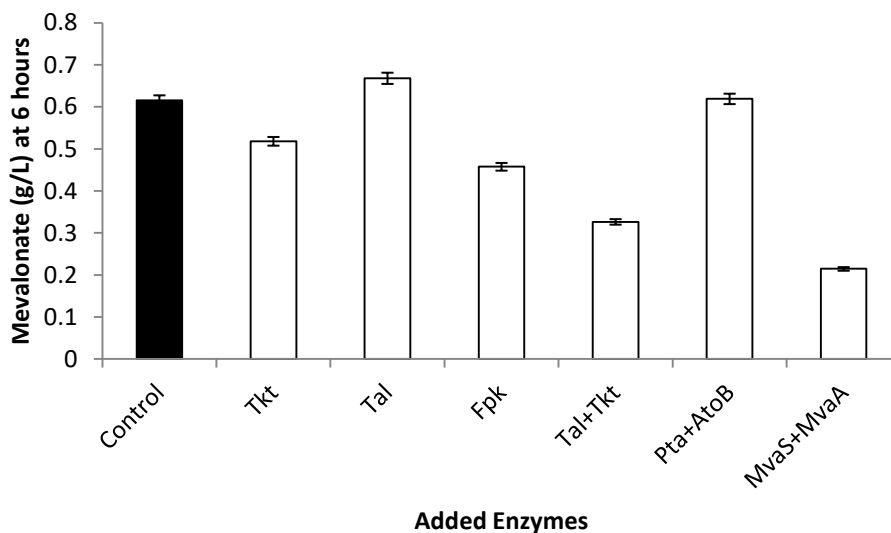


Figure 11 Doubling of various enzymes to test any further production of mevalonate with data taken at 6 hours. Tal shows slight increase of mevalonate production compared to control, but not a significant increase.

Although mevalonate production was observed with increase in Tal, the increase is most likely attributed to the increase of glycerol amount from the added enzyme. No further enzyme adjustment was conducted. After adjusting the enzyme levels, we were able to achieve greater mevalonate production needed to achieve a higher carbon yield. The final enzyme and the respective amounts used are listed in the method section. For clarification, no glycerol is added to *in vitro* production since there is sufficient glycerol from the storage of the enzymes for the mevalonate production.

To calculate carbon yield, we measured the glycerol consumption and mevalonate production between two time points and used the following Equation 1:

$$\text{Carbon Yield (\%)} = \frac{\frac{(\Delta \text{ Production Mevalonate}) \left[\frac{\text{g}}{\text{L}} \right]}{\text{MW of Mevalonate}}}{1 \text{ mol of Mevalonate}} \div \frac{\frac{(\Delta \text{ Consumption Glycerol}) \left[\frac{\text{g}}{\text{L}} \right]}{\text{MW of glycerol}}}{2 \text{ mol of glycerol}} \times 100\% \quad \dots \text{Eq (1)}$$

The division by 1 mol of mevalonate and 2 mol of glycerol in the denominator arises from the fact that 2 glycerol molecules are converted to 1 molecule of mevalonate.

Data for mevalonate production and glycerol production from 0 to 24 hours are represented in Figure 12.

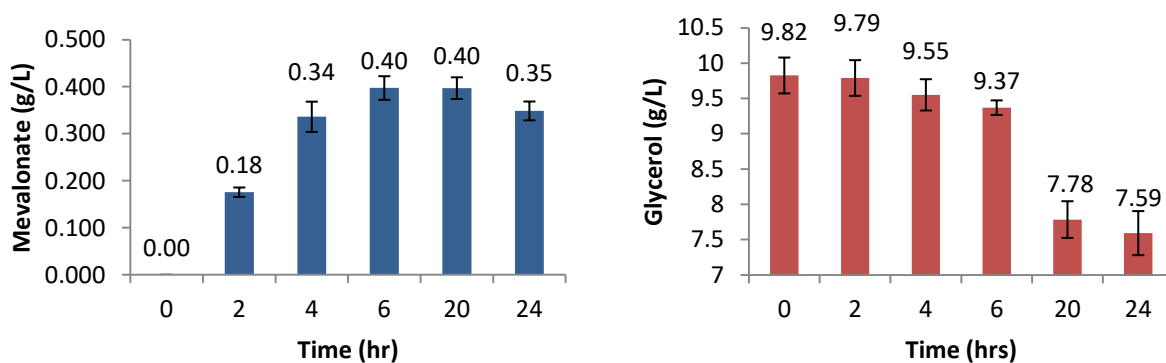


Figure 12 Represents the mevalonate production and glycerol consumption from 0 to 24 hours. Data values represented are average of triplicate data and error bars indicate s.d.

Using the equation to calculate the carbon yield, carbon yield values are summarized in Table 2.

Carbon yield values are calculated with 0 hour as the base value.

Table 2 Represents carbon yield with percentage error. Carbon yield calculated using Equation 1

Carbon Yield	
0 to 2hr	610.66% $\pm$ 220.76%
0 to 4hr	152.41% $\pm$ 52.17%
0 to 6hr	108.51% $\pm$ 25.20%
0 to 20hr	24.65% $\pm$ 25.66 %
0 to 24hr	22.16% $\pm$ 20.59 %

As seen from the above table, at 6 hours the *in vitro* production has been able to measure 108.51% $\pm$ 25.20 % of carbon yield. With this result, we were able to achieve approximately 100% carbon conversion from 2 molecules of glycerol to 1 molecule of mevalonate, and thus validate the feasibility of the proposed glycerol condensation cycle. Past 6 hours, the mevalonate production drops, accounting for the low carbon yield. To find out the cause of mevalonate production drop, tests adding individual enzymes similar to that in Figure 7 had been conducted to the samples that had been incubated at 24 hours to determine the limiting enzyme. There were, however, no improvements in mevalonate production that were observed from this experiment (Figure 13). Another reason why the mevalonate production has dropped may be due to glycerol inhibition of FSA. After publishing this thesis, test to see FSA activity with respect to different incubation time of glycerol will be conducted (0, 2, 4, 6, 20, 24 hours).

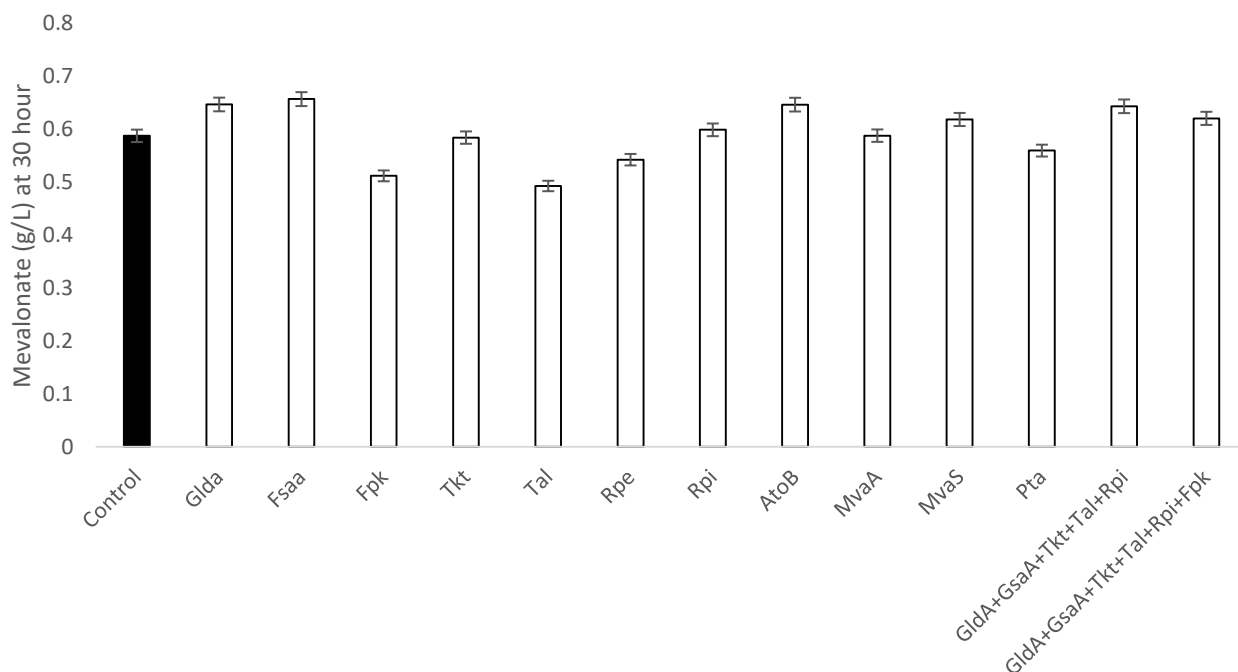


Figure 13 Mevalonate production results with additional enzyme added to 24 hour samples. Compared to control, no enzymes contributed to significant improvement in mevalonate production after 6 hours of enzyme addition. Added amount of enzymes is enzyme level needed for 200 μ L reaction mixture.

Possible reason for why the carbon yield is over 100% at 6 hours and is so high at 2 hours and 4 hours is due to the inaccuracy of glycerol measurement. In the initial phases of the *in vitro* production, carbon yield is highly sensitive to changes in glycerol level. For example, increasing the 0 hour sample glycerol amount by 0.1g/L will lower the carbon yield between 0 to 2 hours from 610.66% to 197.01%. On the other hand, increasing 0 hour glycerol amount by 0.1g/L will only lower the carbon yield between 0 to 6 hours from 108.51% to 93.13%. The change in glycerol level is less sensitive in 0 to 6 hours compared to 0 to 2 hours, which is why we still conclude 100% carbon conversion is achieved. Finding a method for accurately measuring glycerol amount had been repeatedly attempted—such as measuring with GC-MS, using glycerol

analyzer, or trying different dilution factors—but this protocol has yielded the best results as of today. After submission of this master’s thesis, further tests to improve the accuracy of the glycerol measurement will be conducted.

4. Summary and Future Direction

In this work, we constructed a synthetic ATP-independent glycerol utilization pathway to achieve theoretical conversion of Acetyl-CoA. Between 0 to 6 hours, the carbon yield achieved from calculating the consumption of glycerol to production of mevalonate was $108.51\% \pm 25.20\%$, highly verifying the validity of the GCC pathway. The ATP independence property promotes this synthetic pathway for its *in vitro* application by reducing the cofactor cost and the potential ATP inhibition on pathway enzymes. Compared to initial glycerol measurement using GC-MS, HPLC has yielded an increasingly accurate tool. There is, however, room for improved accuracy. As a future direction, methods for further accurate measurements of glycerol will need to be explored such as trying further different dilution factors. In addition, carbon labeling of glycerol would be conducted to ensure that the mevalonate results from the substrate fed to the system. Lastly, the GCC will be demonstrated *in vivo* production for practicality in organisms.

Acknowledgements

This material is based upon research performed in a renovated collaborator by the National Science Foundation under Grant No. 0963183, which is an award funded under the American Recovery and Reinvestment Act of 2009 (ARRA). I would like to thank Tony Wu for constant guidance throughout the process of this project. I would also like to thank Professor Liao for not just my Master's period, but also my undergraduate time period to introduce me to the metabolic engineering field which opened up my vision on this field of science. Lastly, I would like to thank my parents for their constant support throughout my whole life. Without all of these people, I would not have been able to be where I am today. Thank you.

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