Sweet Hydrogen: High-Yield Production of Hydrogen from Biomass Sugars Catalyzed by *in vitro* Synthetic Biosystems

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6/7/2017 (2:45:00 PM)

Project ID PD127

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Overview

Timeline

- Project Start Date: 06/15/2015
- Project End Date: 12/31/2017 (2.5 years), including a half-year no-cost extension from June 30, 2017 to Dec. 31 2017.

Budget

- Total Project Budget: $937,602 (two years)
  - Total Recipient Cost-Sharing: $187,602 (97,882 VT part)
  - Total Federal Share: $750,000
  - Total DOE Funds Spent*: $611,386* As of 3/31/2017

Barriers

Hydrogen production from biomass

AX. Hydrogen Molar Yield

AY. Feedstock Cost

AZ. Systems Engineering

Collaborators

- Virginia Tech (lead)
- University of Georgia

Relevance

Fuel Cell Technologies Office Objective -- Develop advanced biological generation technologies to produce hydrogen with a projected cost of $10.00/gge at the plant gate by 2020.

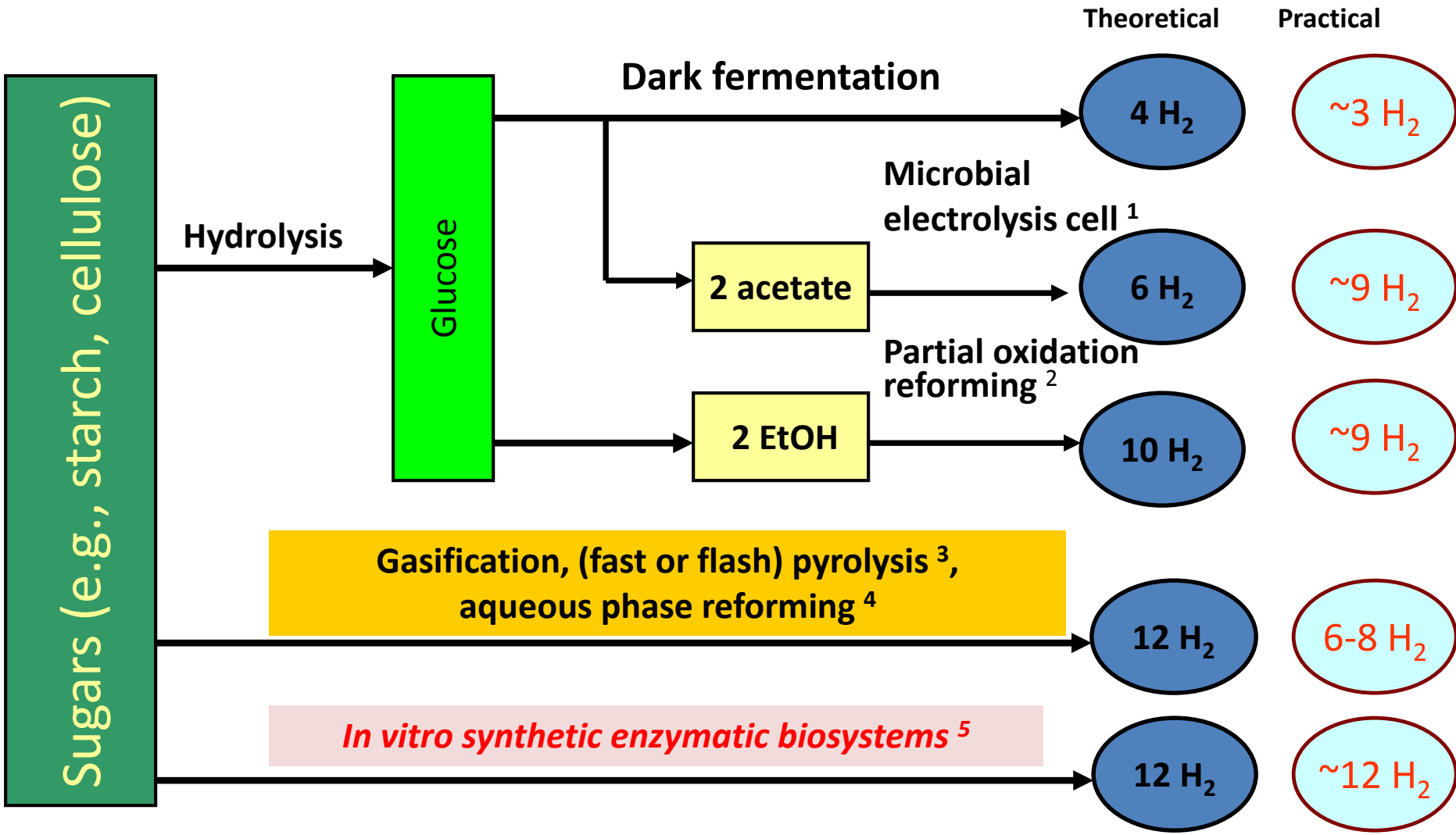
Our novel approach – use renewable sugars (e.g., biomass sugars or starch) to split water to produce H_2 catalyzed by enzyme cocktails

$$C_6H_{10}O_5 + 7\ H_2O = 12\ H_2 + 6\ CO_2$$

- Low-carbon production in terms of entire life cycle
- High-purity hydrogen generated (no CO)
- Mild reaction conditions (1 atm, 20-90°C, pH 7.5, aqueous phase)
- Local resources for distributed hydrogen small-size production (e.g., 1,500 kg/day)
- Highest biological hydrogen generation rates (e.g., **550 mmole H_2/L/h**, now)

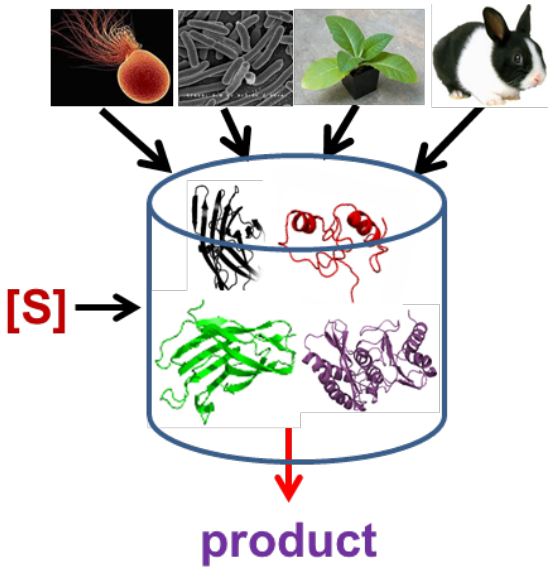
Targets	Units	June 2017 Target	Dec. 2017 Target (estimated)	Year 2020 Target (Plant gate)
Production cost	$/kg H_2	1000	10	10.00 (year 2020)
Productivity	mmole H_2/L/h	750	750	2,000
Reactor volume	L of reactor	1	1	65,000*
				*1,500 kg H_2 per day

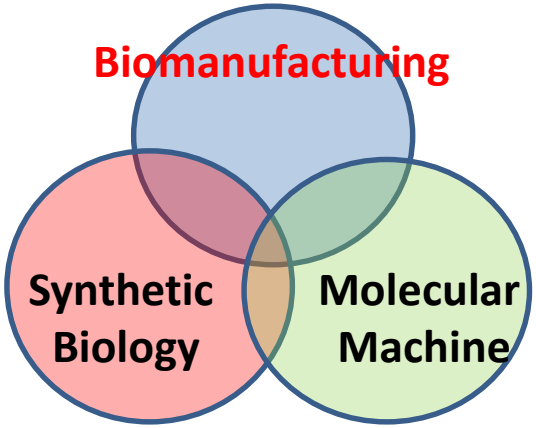
Production of H_2 from sugars (overview)



1. Logan et al. 2007. PNAS 104:18871.
2. Schmidt. 2004. Science. 303:993
3. Schmidt. 2007. Agnew Chem. 46: 586
4. Dumesic et al. 2002. Nature 418:964
5. Zhang et al. PLoS One 2007; ChemSusChem 2009; Angew Chem 2013; Metab Eng 2014; PNAS 2015.

Advanced biotransformation catalyzed by *in vitro* synthetic enzymatic biosystems





Unique features

A disruptive platform, against the dominant biotech paradigm.

- High product yield
- High energy efficiency (oxygen-free biotransformation)
- Fast volumetric productivity
- Broad (mild) reaction conditions
- Easy product separation
- High product titer
- Tolerance of toxic compounds (e.g., substrate, product, solvent)
- Great engineering flexibility
- Easy process control and scale-up
- Low capital investment (CapEx)
- Accomplish non-natural bio-reactions

Zhang Y-HP*. 2017. Biomanufacturing: History and Perspective. *J. Ind. Microbiol. Biotechnol.* 44: 773-784

Approaches/Milestones

(*As of 3/31/2017)

FY 15 Q4	FY 16 Q1	FY 16 Q2	FY 16 Q3	FY 16 Q4	FY 17 Q1	FY 17 Q2	FY 17 Q3	FY 17 Q4	FY 18 Q1

Task 1.1. Co-expression of multiple enzymes in one host	(FY17 Q1)	100%
Task 1.2. Two redox enzymes on biomimics at 1 U/mg	(FY17 Q3)	80%
Task 1.4. H_2 production cost of \$10/kg H_2 by H2A model	(FY17 Q3)	75%
Task 2.2. Data fitting and validating of rate-limiting steps	(FY16 Q4)	100%
Task 2.3. Construction of artificial electron transport chains	(FY17 Q3)	100%
Task 2.4. Construction of five synthetic enzyme complexes	(FY17 Q3)	100%
Task 3.1. High-density of protein expression in *E. coli*	(FY17 Q3)	100%
Task 3.2. Mass production of hydrogenase (SH1) (UGa)	(FY17 Q3)	70%
Task 3.3. 1000-mL level demonstration	(FY17 Q3)	90%

This reporting period (Dec 2017, a final deliverable)

- **1,000-fold volume scale-up (1 L reactor) with 5-fold increase in H_2 peak production rate (i.e., 750 mmol H_2/L/h or 1.5 g H_2/L/h)**

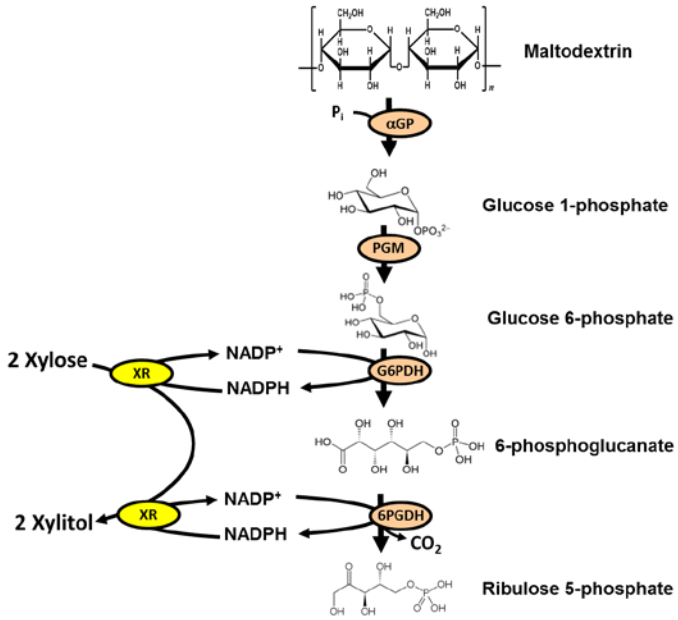
Accomplishments and Progress:

Responses to Previous Year Reviewers' Comments

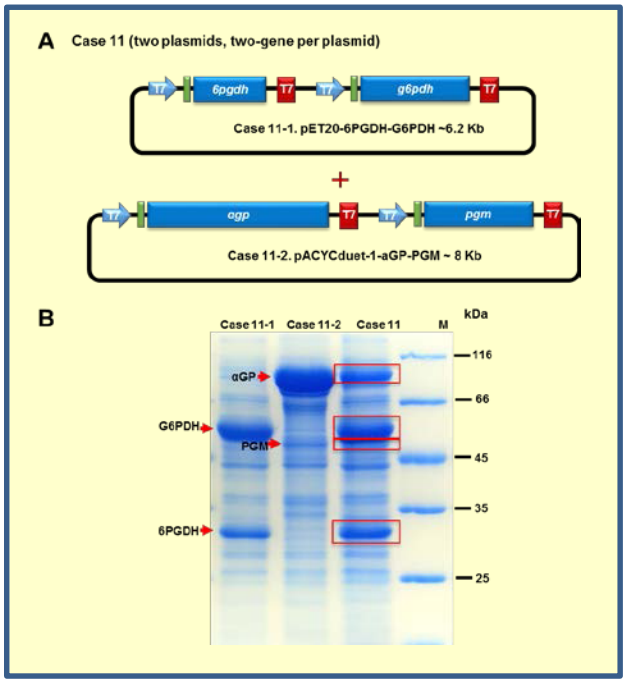
FY16 reviewers' comments	FY17 response to comments
Substrate cost. *"This in vitro approach will likely be effective only for very clean sugars/starches and so will have somewhat limited greenhouse gas benefits when compared to a lignocellulosic system."*	Starch ($0.30/kg) is the cheapest sugar. Glucose made from starch is cheaper than that made from biomass. It can use biomass sugars in the presence of toxic compounds (PNAS 2015). Also, we will make artificial starch from biomass (PNAS 2013) and via artificial photosynthesis (Energy Sci. Eng., 2013). Starch may be the best solar fuel and hydrogen storage compound.
Enzyme production cost. " *With the synthesis of the enzymes and identification of challenges/solutions with the cofactors, the project appears to be off to a promising start in terms of meeting the H_2 volume objectives."*	Current production costs of recombinant thermophilic enzymes in *E. coli* are $50/kg dry enzyme or lower (JIMB 2017). It may be decreased to $10-20/kg, like amylase, protease (JIMB 2017). This first industrial biomanufacturing example with in vitro biosystem has been established in China (B&B 2017).
Coenzyme replacement and costs (ibid)	Replacement of NADP with NMN and NAD-conjugate could decrease H_2 production costs to less than $10/kg H_2, when enzyme costs and stability are addressed.
H_2 production rates. "how *long* certain hydrogen productivity rates could be achieved"	We demonstrate up to 3-h high-speed H_2 production (i.e., 1 g H_2/L/h) in a batch mode (see following PPT).

Accomplishments: Four-enzyme coexpression

Four enzymes: αGP, PGM, G6PDH & 6PDGH



Four enzymes in two plasmids



Chen et al. 2017. Appl. Microbiol. Biotechnol.:Doi: 10.1007/s00253-017-8206-8.

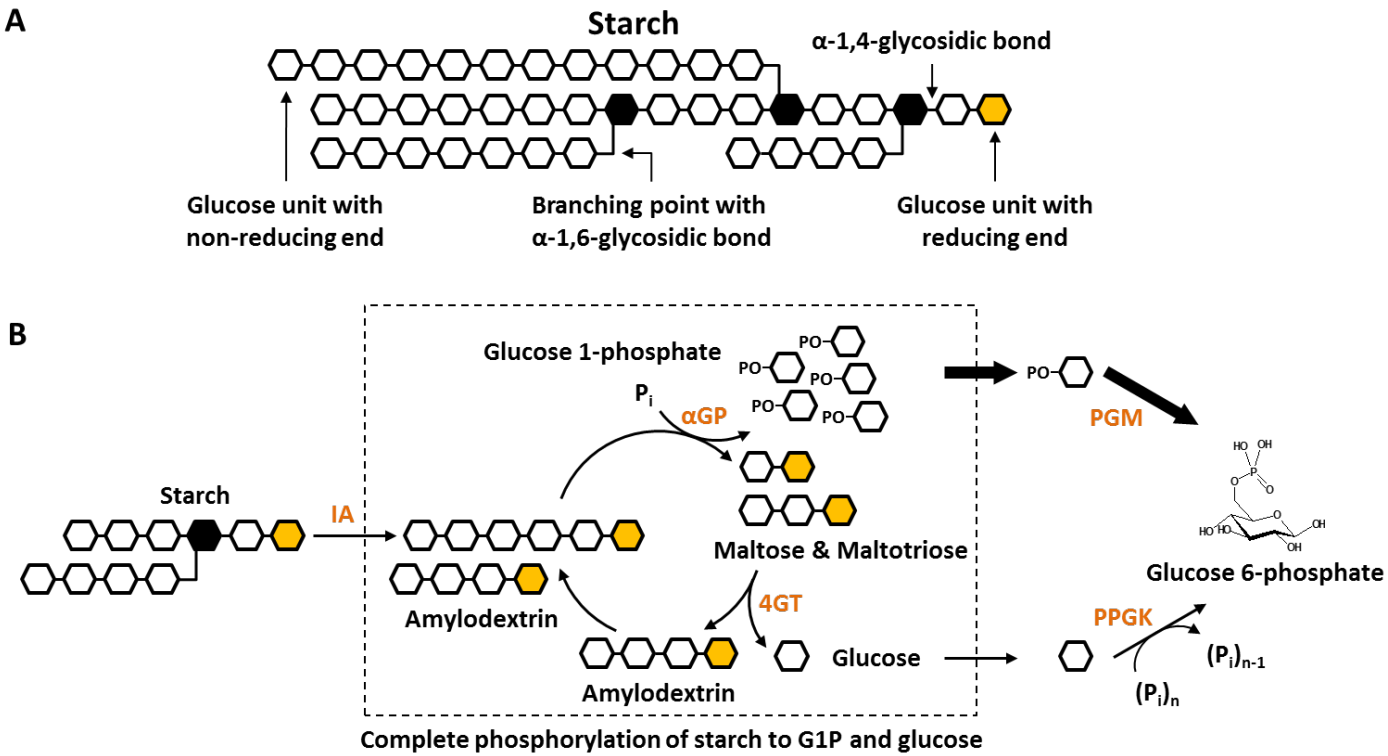
Table 3 Comparison of four-enzyme co-expression cases in *E. coli* and the activity based on xylitol generation

Case name	Protein expression percentage (%)					Apparent enzyme activity (U/mL)[a]				Overall activity (mM)[b]
	αGP	PGM	G6PDH	6PGDH	Sum	αGP	PGM	G6PDH	6PGDH	
Case 8	30	6.2	4.2	1.6	42	2.8	16	0.29	0.10	15.48
Case 9	28	6.9	3.9	1.3	40	2.6	18	0.27	0.083	15.27
Case 10	16	5.4	3.6	17	42	1.5	14	0.25	1.1	16.67
Case 11	17	0.75	28	20	66	1.6	1.9	1.9	1.3	24.58

We found out that the best strategy (two different strength plasmids, each has two genes encoded) to precisely control four enzyme expression levels in *E. coli* BL21(DE3).

Achievements: Complete starch utilization for H_2 generation

Enzymatic starch phosphorylation without ATP

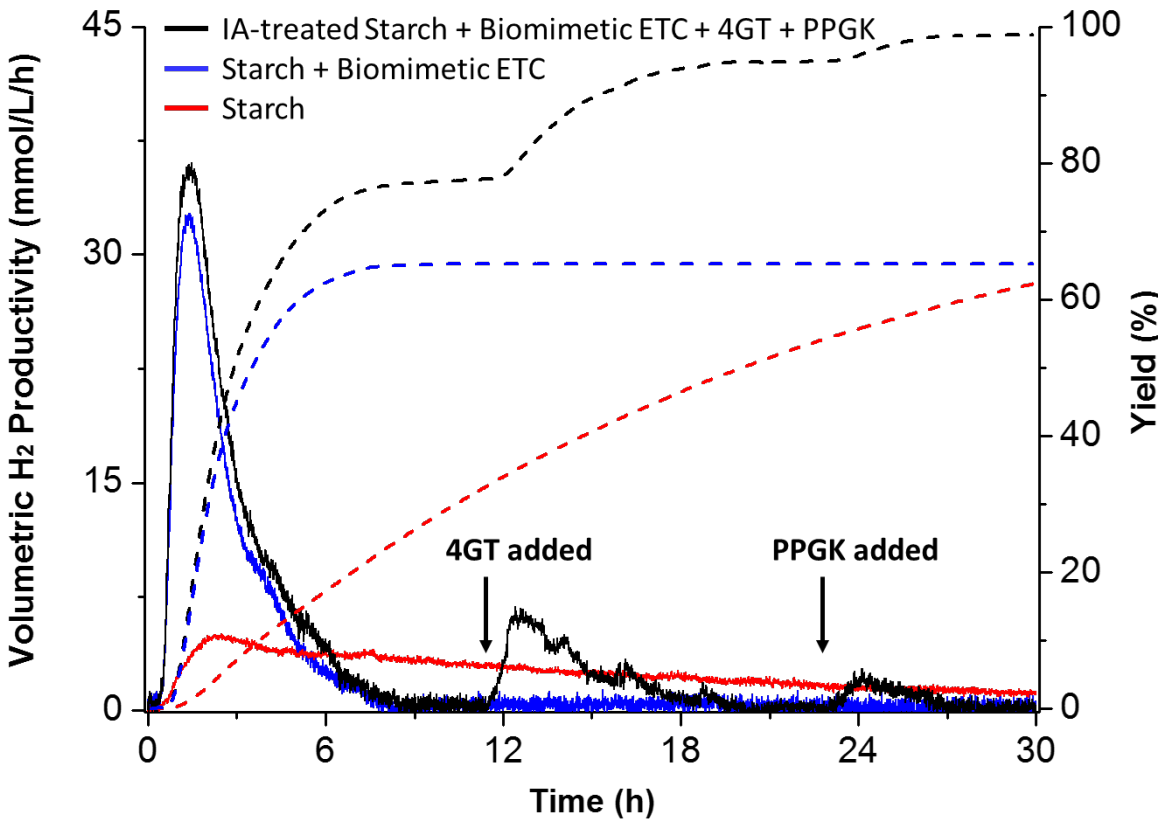


- **Alternative phosphorolysis of starch, yielding G6P (better than simple hydrolysis).**
- **Use isoamylase to debranch branched starch (amylopectin) to increase H_2 yield by 30%)**
- **Use 4-glucanotransfer (4GT) to utilize maltose to increase H_2 yield about 4%.**
- **Polyphosphate glucokinase (PPGK) to utilize glucose to increase yield about 4%.**

Kim et al. 2017. Complete enzymatic phosphorylation of starch for green hydrogen gas production at theoretical yield by in vitro metabolic engineering. (submitted).

Achievements: Complete starch utilization for H_2 generation (2)

$$C_6H_{10}O_5 + 7\ H_2O = 12\ H_2 + 6\ CO_2$$



Conditions:
50°C, pH 7.5

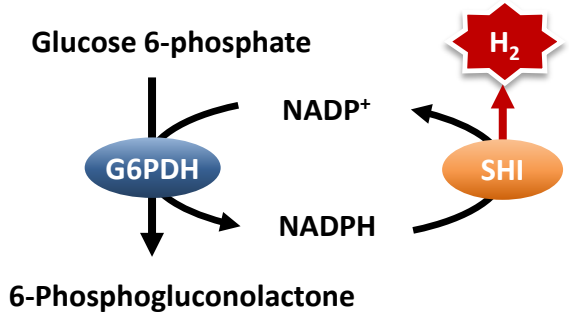
For the first time we can utilize all glucose units of starch without ATP for H_2 generation.

$0.30/kg starch = $2.02/kg of H_2 (our goal is $10/kg delivered H_2).

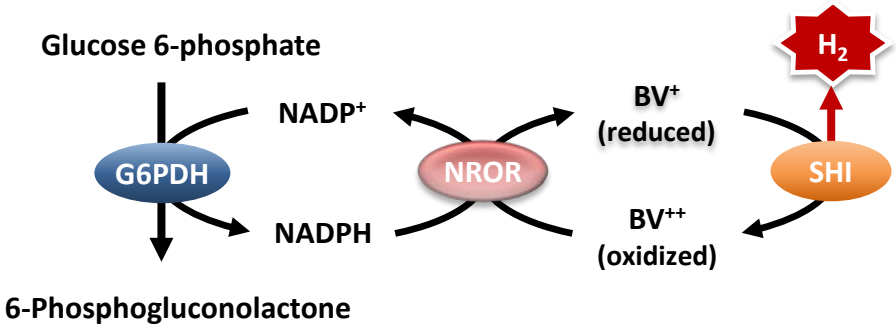
Kim et al. 2017. Complete enzymatic phosphorylation of starch for green hydrogen gas production at theoretical yield by in vitro metabolic engineering. (submitted).

Accomplishments: Increasing Rxn by artificial electron transport chains

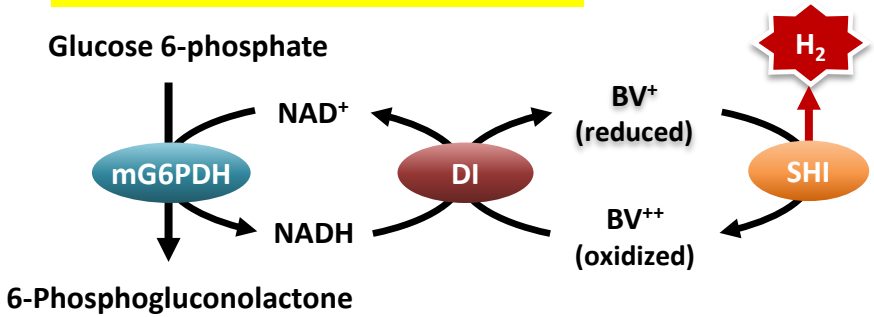
a Original design (2007)



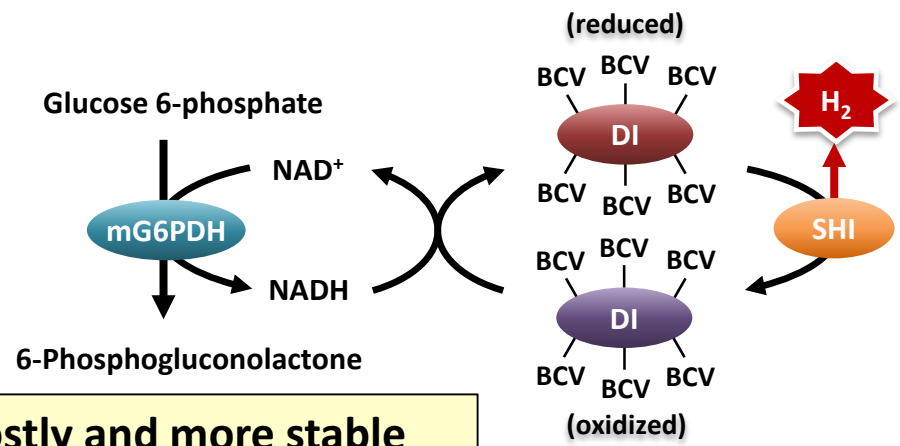
b Revised 2016



c NAD-based PPP 2017



d NAD-based PPP + conjugate 2017



- **Decreasing production costs by using less costly and more stable coenzymes instead of $NADP^+$.**

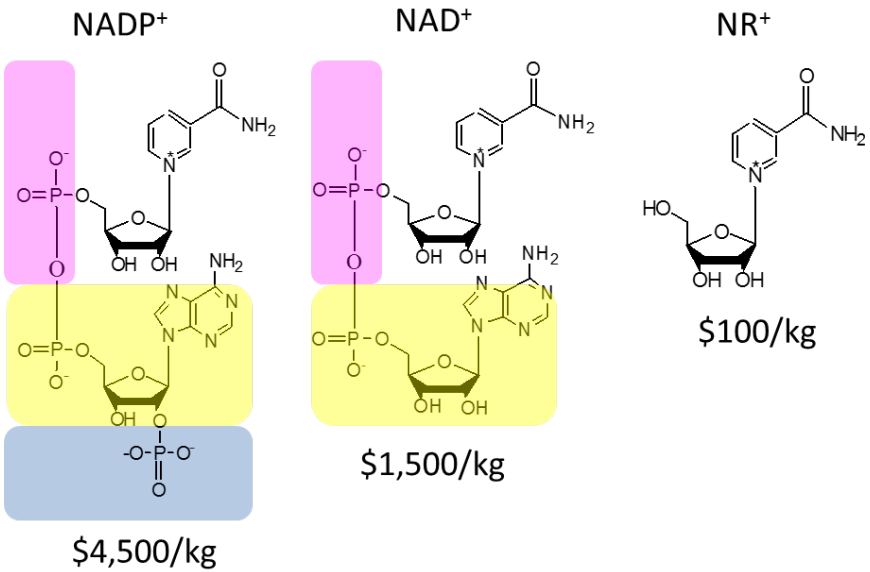
Kim et al. 2017. Ultra-high speed production of biohydrogen gas *in vitro*. (submitted).

Progress: Replace NAD(P) of dehydrogenases with NAD & NMN

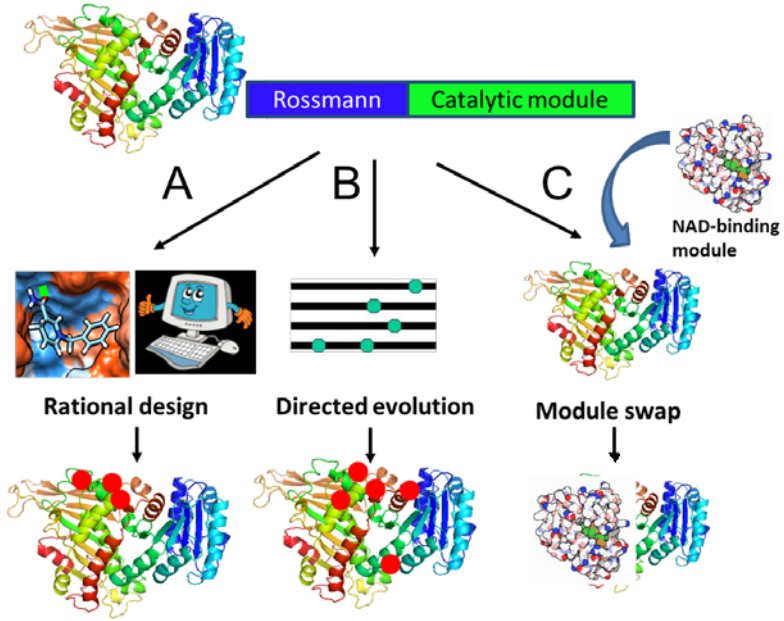
Glucose 6-phosphate dehydrogenase (G6PDH)
6-phosphogluconate dehydrogenase (6PGDH)

Specific activity order
+++++ (NADP), ++ (NAD), - (NMN), - (NR)

Comparison of coenzymes



Coenzyme engineering



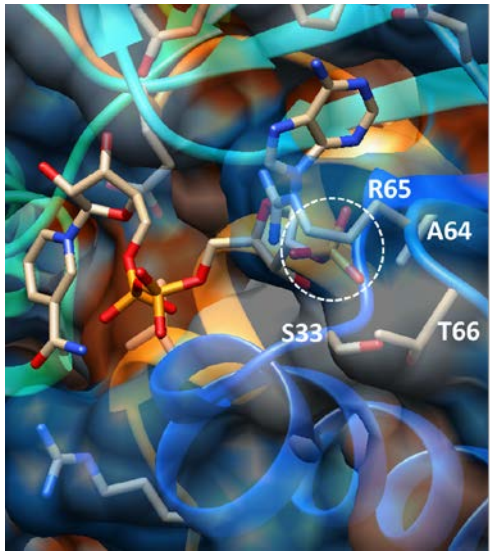
NAD and nicotinamide riboside (NR) are better than NAD(P)

- Less costly
- More stable
- Small size – better mass transfer

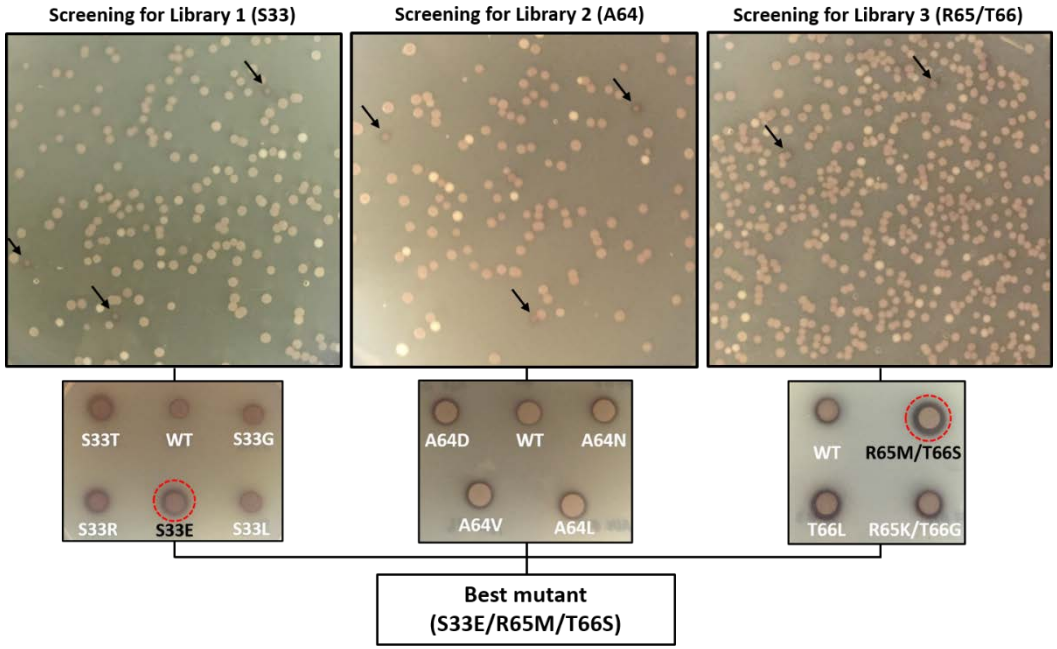
- **We have three strategies to change coenzyme preference of dehydrogenases**
- **New area – very high risk.**

Achievement: TmG6PDH coenzyme from NADP to NAD

Identification of key amino acids followed by high-throughput screening



Autodock module



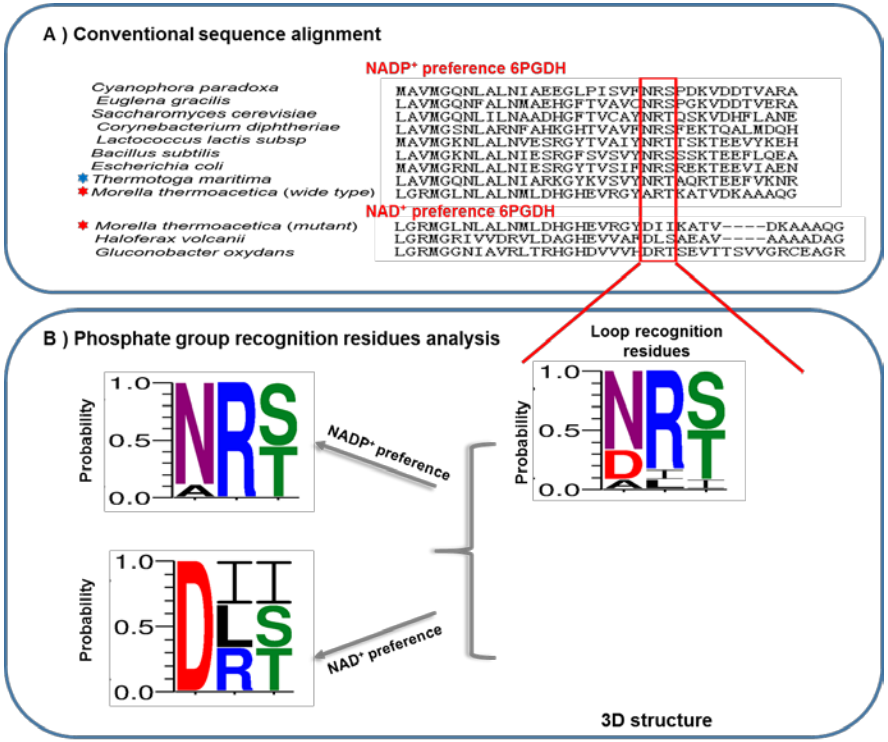
Redox dye-based screening plate

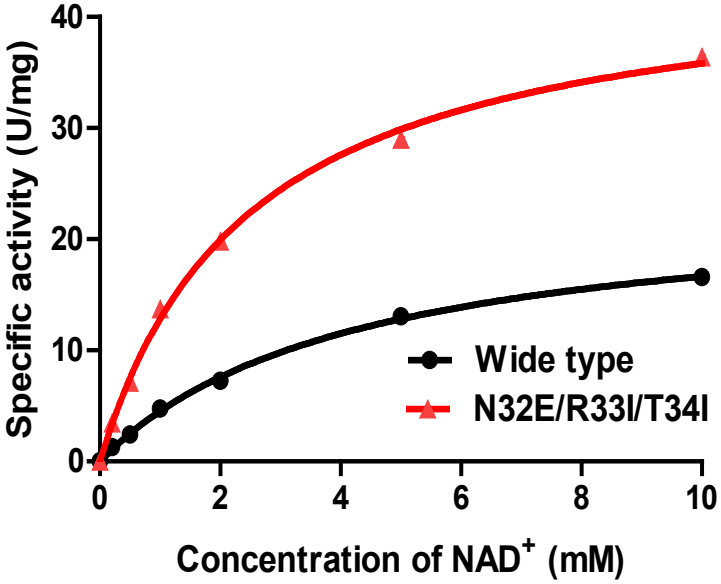
The best mutant TmG6PDH (S33E/R65M/T66S) exhibited a preferred coenzyme from $NADP^+$ to NAD^+.

Huang R, Chen H, Zhang Y-HP*. 2016. High-throughput screening of coenzyme preference change of thermophilic 6-phosphogluconate dehydrogenase from NADP+ to NAD+. Scientific Reports 6:32644. Kim et al. 2017. Ultra-high speed production of biohydrogen gas *in vitro*. (submitted).

Achievement: Tm6PGDH coenzyme from NADP to NAD

Rational design



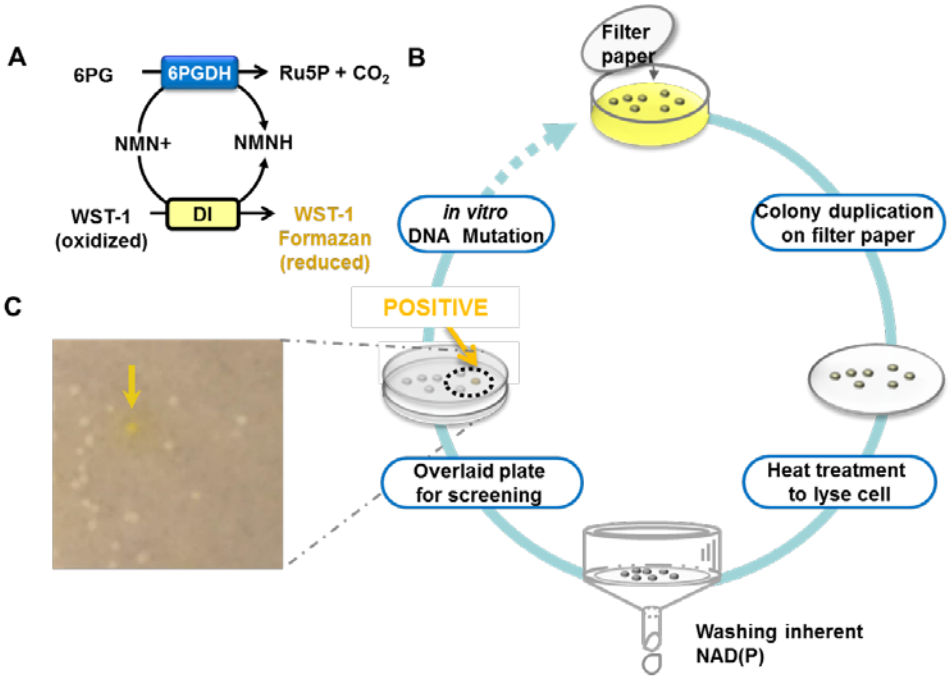


- Via rational design (amino acid alignment and homologous molecule structure), we identified the three amino acids binding to the phosphate group of NADP.
- The best mutant TmG6PDH (N32E/R33I/T34I) exhibited a 6.4 × 10^4-fold reversal of the coenzyme selectivity from $NADP^+$ to NAD^+.

Chen et al. 2016. Coenzyme engineering of a hyperthermophilic 6-phosphogluconate dehydrogenase from NADP+ to NAD+ with its application to biobatteries. Sci. Rep. 6:36311.

Progress: Coenzyme engineering toward biomimics

Novel screening method with minimal interference from intracellular NAD(P)

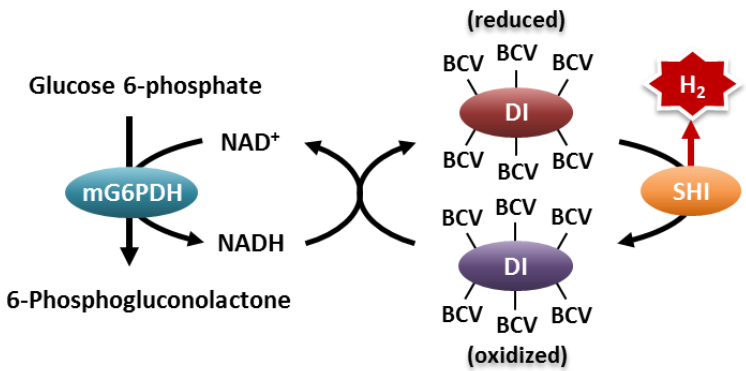


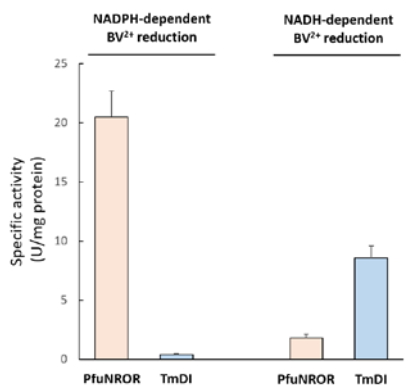
- **We conducted three-round site-directed mutagenesis and one-round whole gene sequence mutagenesis, yield the best mutant Tm6PGDH having a specific activity of more than 1 U/mg on NMN.**
- **The best mutant has up to 10 amino acid changes (e.g., A11G/R33I/T34I/D81I/T82I/Q86I/D294V/Y383C/N387S/A447V).**
- **Met one of milestones of coenzyme engineering (> 1 U/mg on NMR).**

- **We are repeating our successful efforts in the other dehydrogenase – TmG6PDH.**

Achievement: First NAD-based pentose phosphate pathway

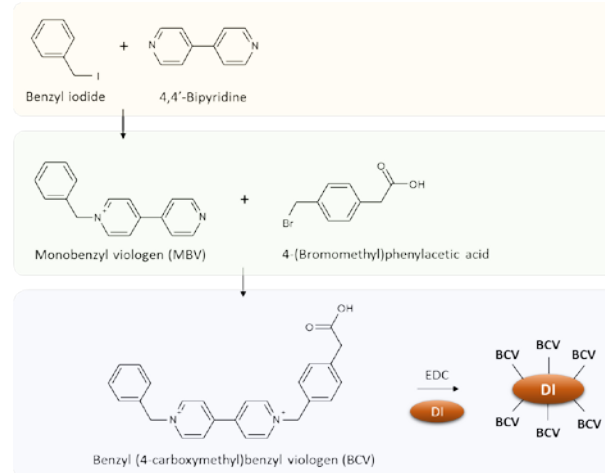
NAD-based PPP + conjugate 2017

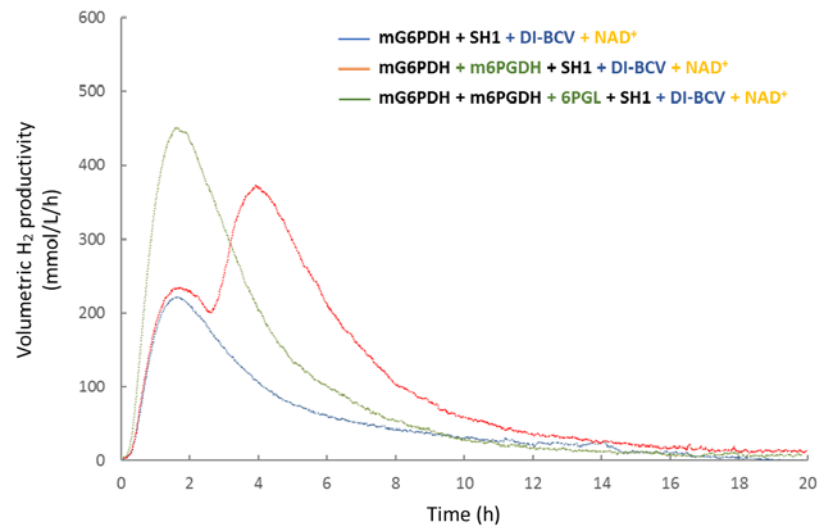




Discover a new diaphorase (instead of NROR) for NAD-based ETC, increasing Rxn by 5-fold

Making DI-BCV conjugate



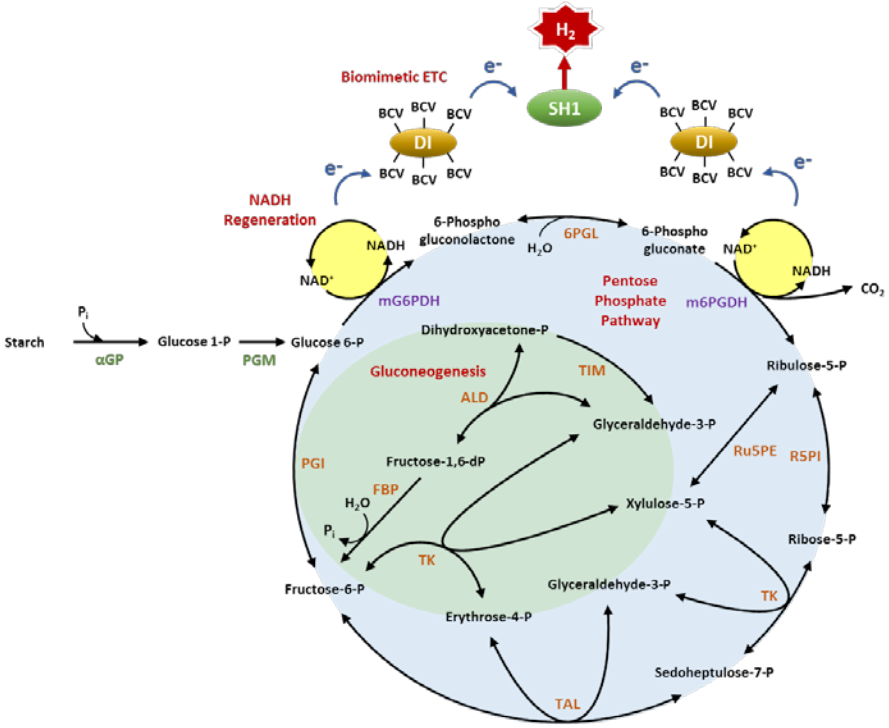


Doubling Rxn by 2-fold by using DI-BV conjugate.

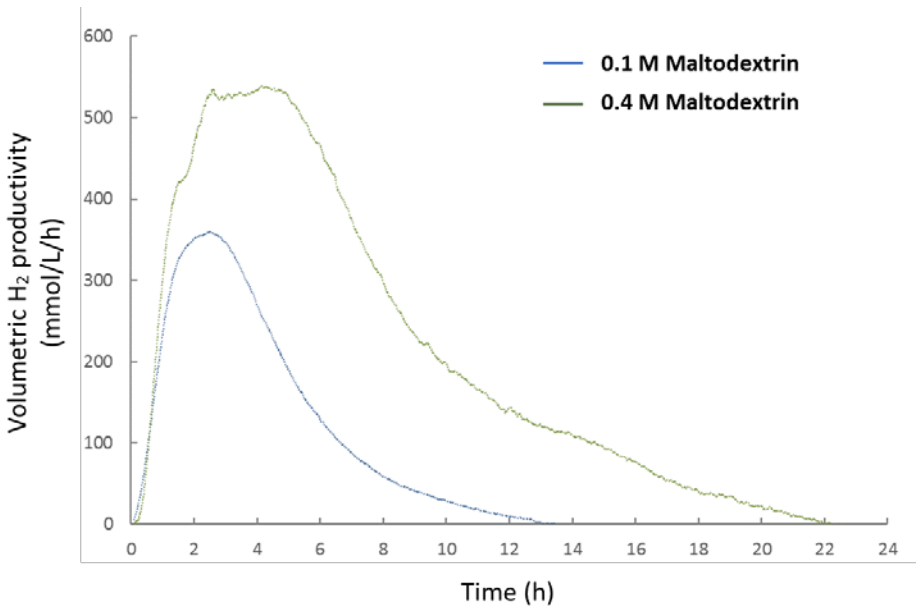
Kim et al. 2017. Ultra-high speed production of biohydrogen gas *in vitro (submitted)*

Achievements: Increasing Rxn to 1 g H_2/L/h

Revised pathway based on NAD and BV-DI conjugate



Ultra-rapid in vitro H_2 generation



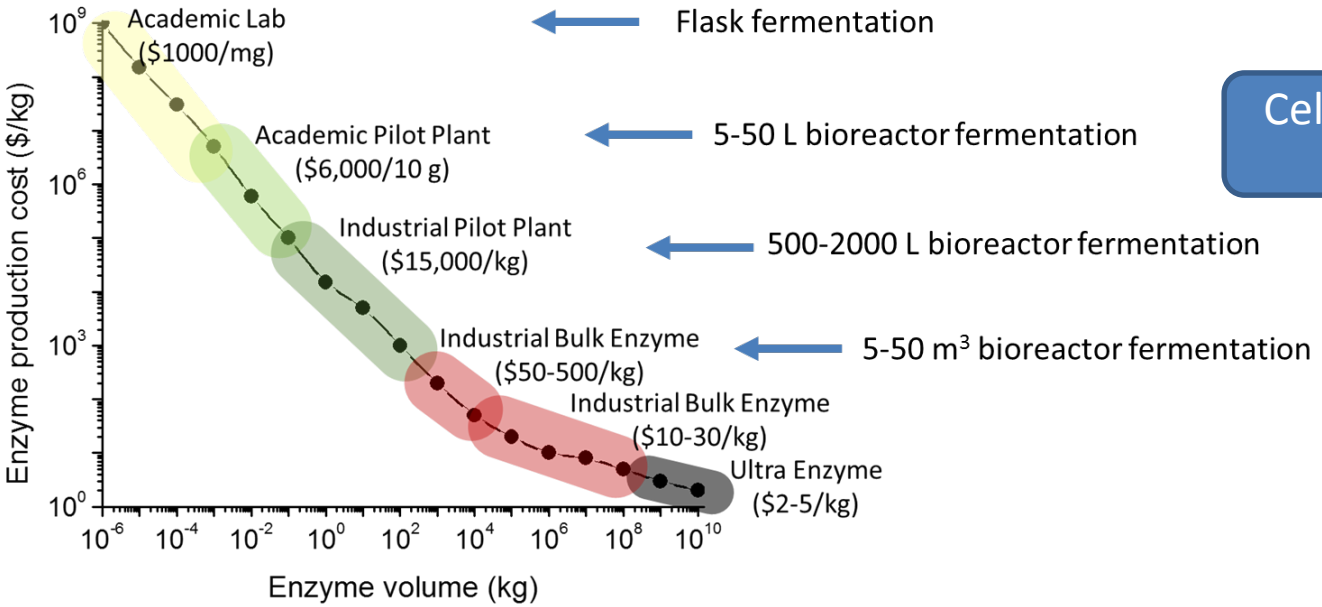
Conditions: 80°C, pH 7.5

- **Maximum H_2 generation rate = 530 mmole H_2/L/h = 1.06 g H_2/L/h**
  = 272 L of H_2/L/d = ~8 g glucose consumption/L/h
- **A plant producing 1,500 kg H_2/day = ~70 m^3 (anaerobic digester, beer fermenters)**

Kim et al. 2017. Ultra-rapid production of biohydrogen gas *in vitro*. (submitted).

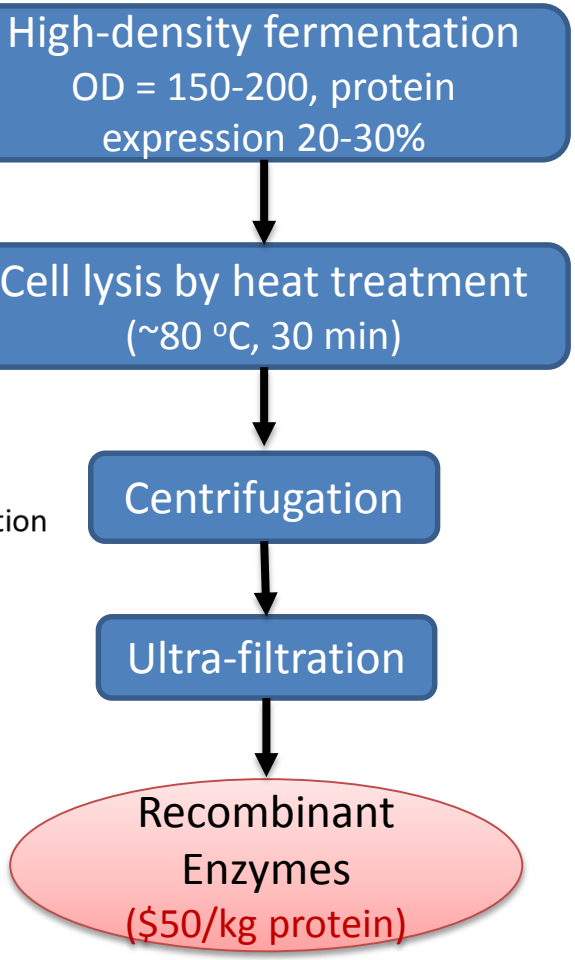
Progress: Scale-up of high density *E. coli* fermentation

Comparison of academic & industrial enzyme production cost



Enzyme costs are NOT a showstopper for *in vitro* synthetic biosystems.

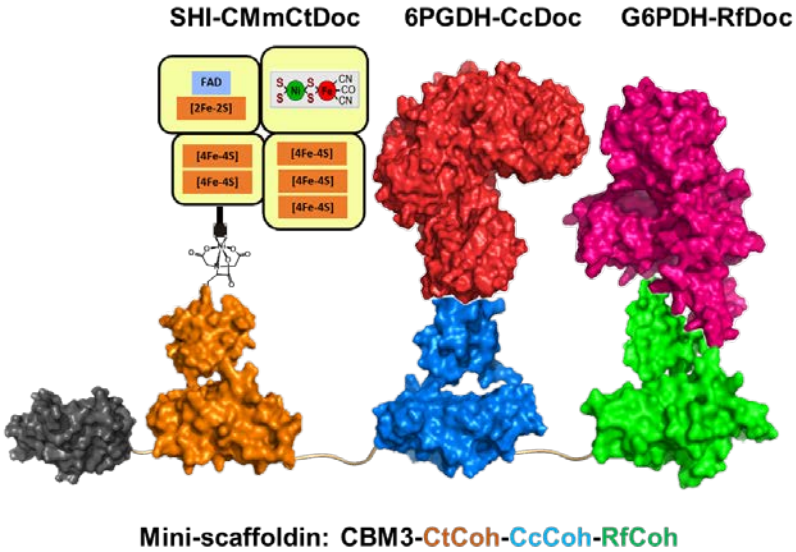
Thermoenzyme prod. & purif.

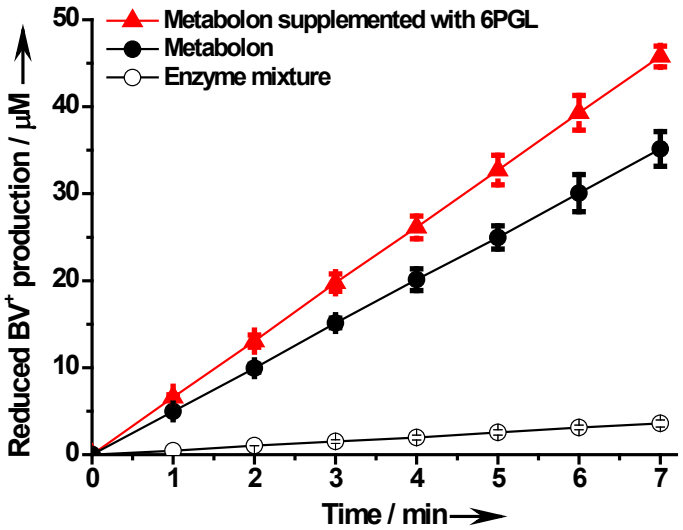


Zhang et al. 2017. Biomanufacturing: History and Perspective. J. Ind. Microbiol. Biotechnol. 44: 773-786.
You et al. 2017. An in vitro synthetic biology platform for the industrial biomanufacturing of myo-inositol from starch. Biotechnol. Bioeng.:DOI: 10.1002/bit.26314.

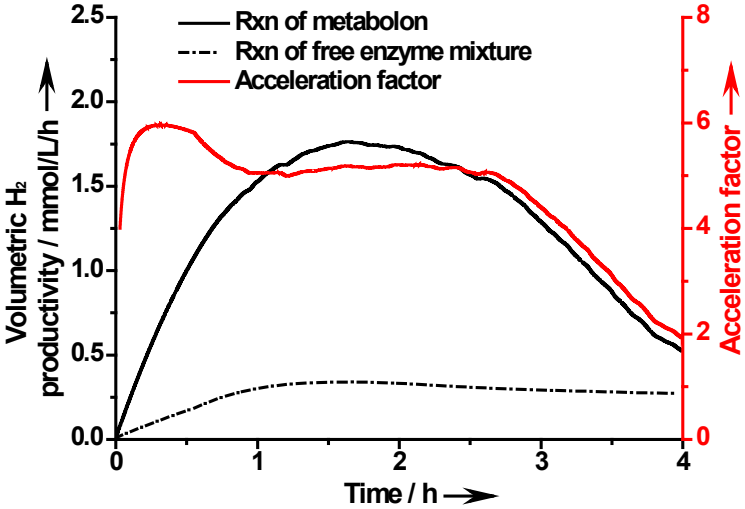
Achievements: Electron channeling in metabolon

Self-assembling metabolon





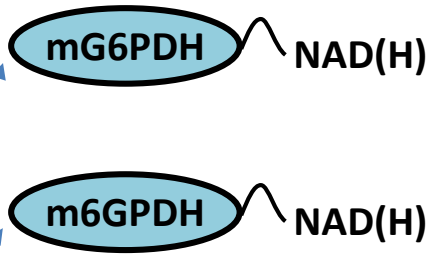
- The metabolon exhibited an initial reaction rate 12.3 times that of the enzyme mixture based on reduction of oxidized benzyl viologen.
- The hydrogen generation rate catalyzed by the metabolon was approximately 5.6 times of that of the enzyme mixture.
- Such reaction rate enhancements suggested strong electron channelling among the adjacent redox enzymes of the metabolon.



Chen et al. 2017. Accelerating electron channeling in the self-assembling metabolon containing two dehydrogenase and NiFe-hydrogenase (submitted)

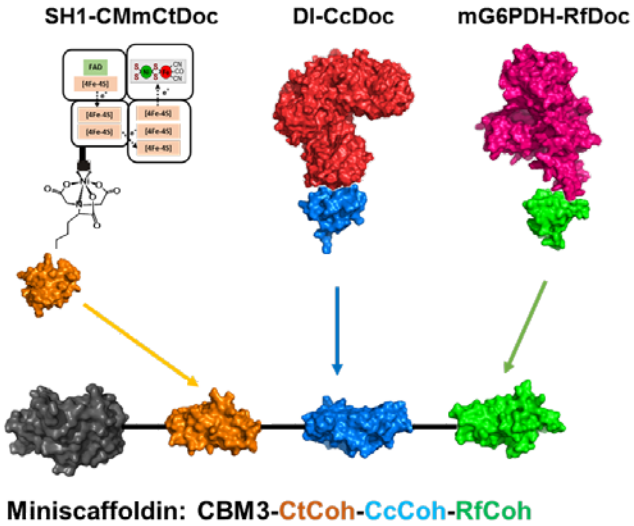
Progress: More metabolons constructed

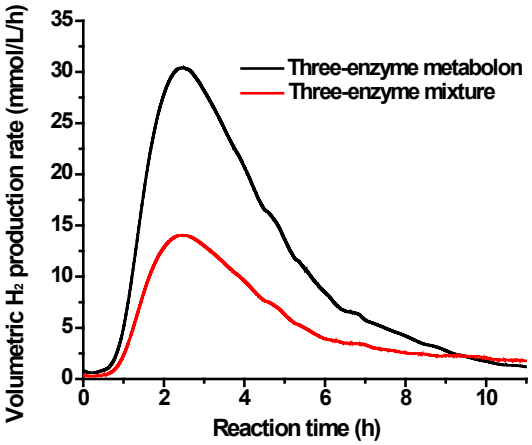
Dehydrogenase-NAD conjugates



- Facilitate rapid electron transfer by 2-3 fold
- Decrease NAD use
- Stabilize NAD by 20-fold than free NAD.

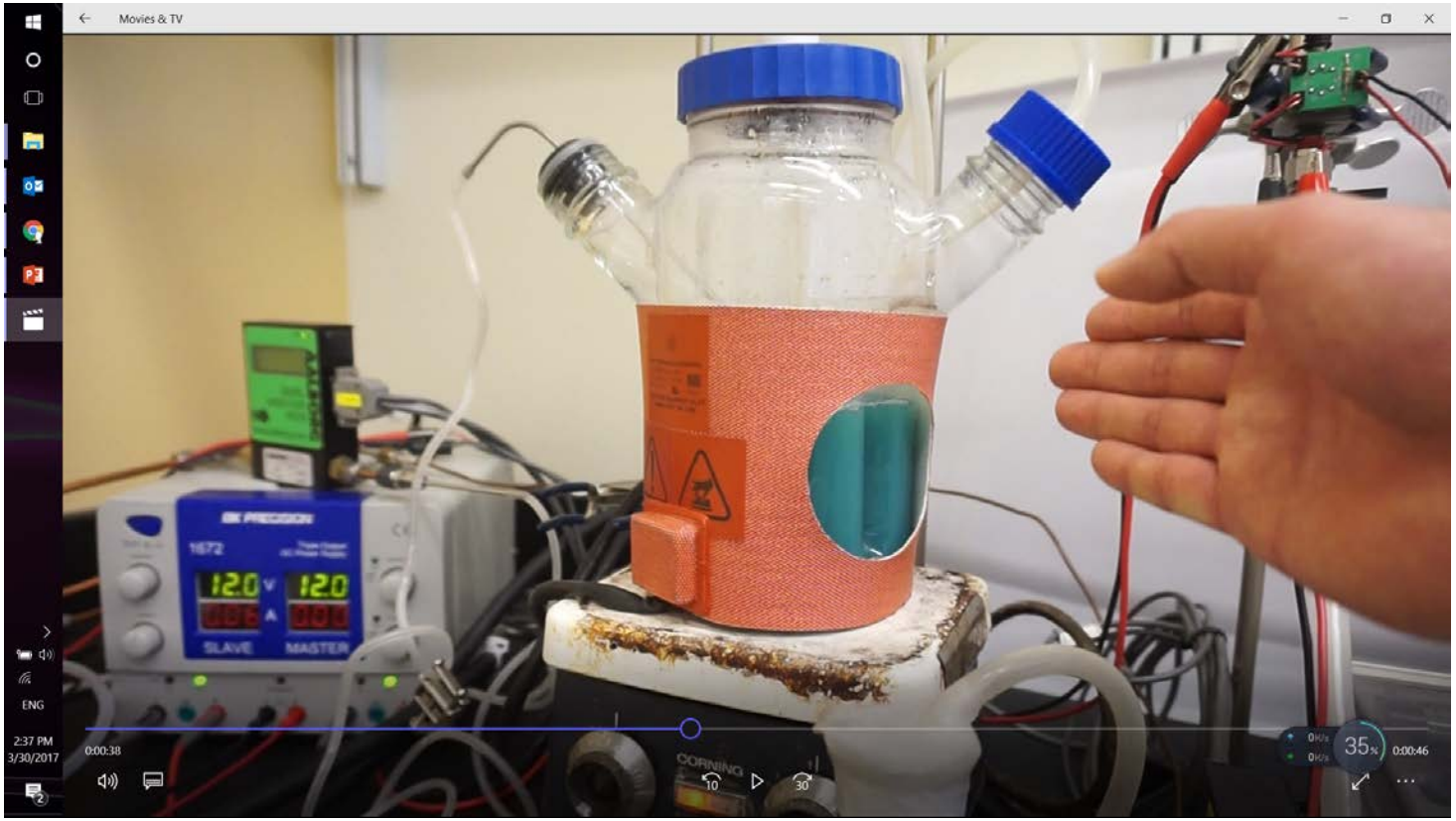
Metabolon featuring ETC





- Increase H_2 generation rate by ~2-3 fold
- Stability of metabolon at high temperature needs to be addressed
- A possible solution is chemical cross-linking.

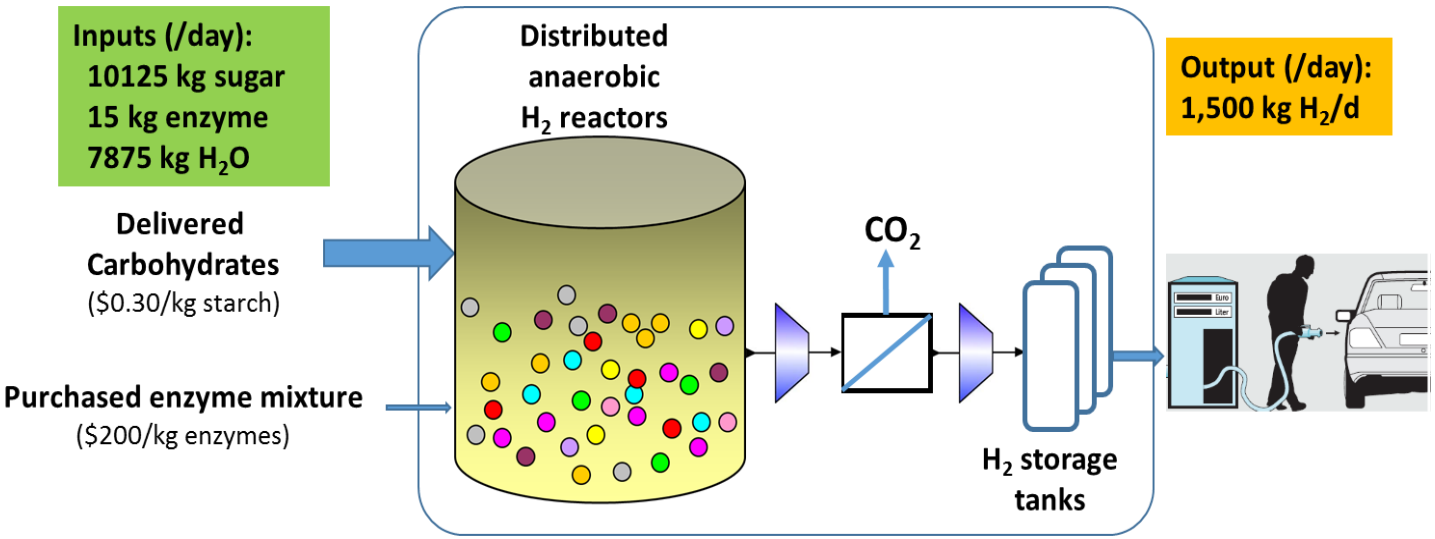
Progress: 1-liter H_2 dry run production



- **We have finished 10-mL experiment in June 2016, meeting the Go/No-Go milestones of Phase I.**
- **We have tested 1-L dry run H_2 production as shown above (at 80°C).**
- **We will conduct 1-L demonstration by integrating latest enzyme complexes and new SH1 by Dec. 2017.**

Progress: H_2 production cost analysis

Distributed green hydrogen production stations (1500 kg H_2/day)



Key inputs

(1) 0.30/kg starch ($0.20/kg in the future)
(2) Enzyme cocktail = ~$200/kg
(3) 1 kg of enzyme (TTN) = 100 kg of H_2
(4) Coenzyme ($/kg) = 500/kg NADP; 1500/kg NAD; $500/kg NMN; and $250/NR
(5) Coenzymes' total turn-over numbers: 20 k for NADP & NAD; 400 k for NAD-conjugate, NMN & NR

Cost (/kg H_2) = starch ($2.025) + enzymes ($2.00) + coenzyme (not including CapEx & OpEx)

Coenzyme	Cost ($/kg H_2)
NADP	$87.77
NAD	$28.90
NAD-conjugate	$ 5.35
NMN	$ 4.23
NR	$ 4.09

Collaboration

The University of Georgia

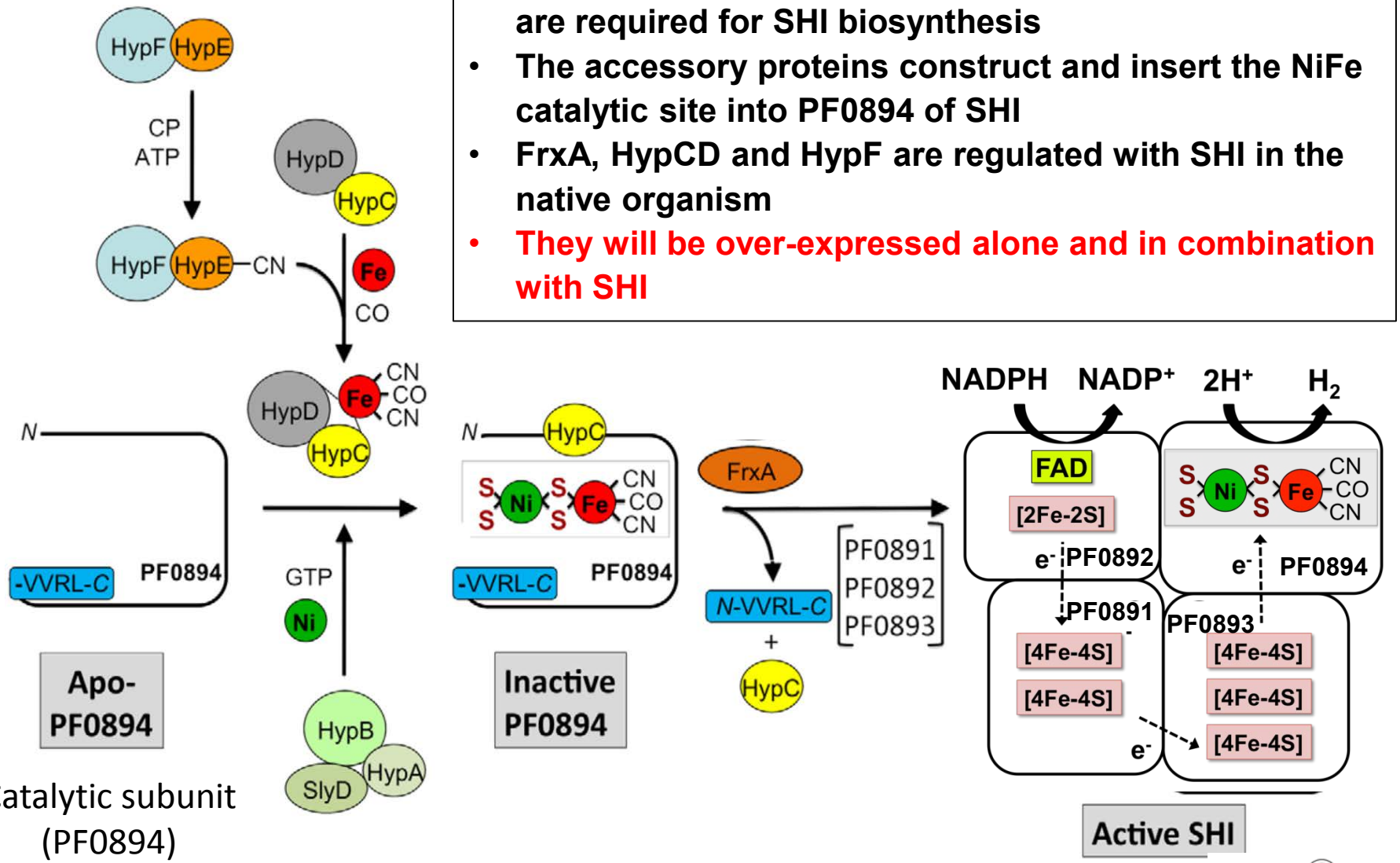
- Subcontract – Co-PI (Prof. Mike Adams) at University of Georgia

- Focus on low-cost mass production of hyperthermophilic Fe-Ni soluble hydrogenase I (*P. furiosus* SH1) without a discount of specific activity of SH1.

- Related to **Objective 1** (decrease hydrogen production costs for enzymes) and **Objective 3** (scale-up of enzymatic hydrogen production)

Biosynthesis of *P. furiosus* SHI is Complex and Requires Eight Accessory Genes

- Eight accessory genes (*hypABCDEF*, *frxA* and *slyD*) are required for SHI biosynthesis
- The accessory proteins construct and insert the NiFe catalytic site into PF0894 of SHI
- FrxA, HypCD and HypF are regulated with SHI in the native organism
- They will be over-expressed alone and in combination with SHI

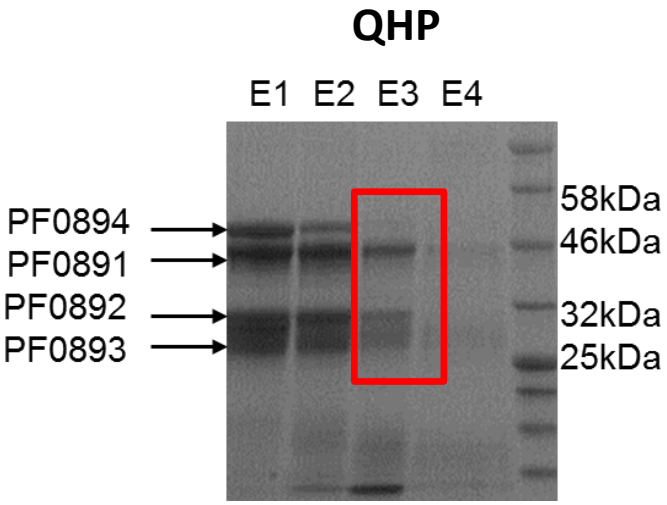


Catalytic subunit (PF0894)

Scale-up: SHI purification from 500 grams of cells

Step	Total Units* (µmol min^{-1})	Total Protein (mg)	Specific Activity (U/mg)	Yield %	Fold Purification
S80	85,807	36,180	2.37	100	1
Ni-NTA	38,147	680	56.1	44	11
Q HP E1	24,301	188	128.9	28.3	54.4
Q HP E2	10,913	136	80.1	12.7	33.8
Q HP E3	1,713	70	24.5	2	10.3
Q HP E4	557	20	27.8	0.6	11.7

*Based on MV-linked H_2 evolution assay



324 mg of SHI was purified

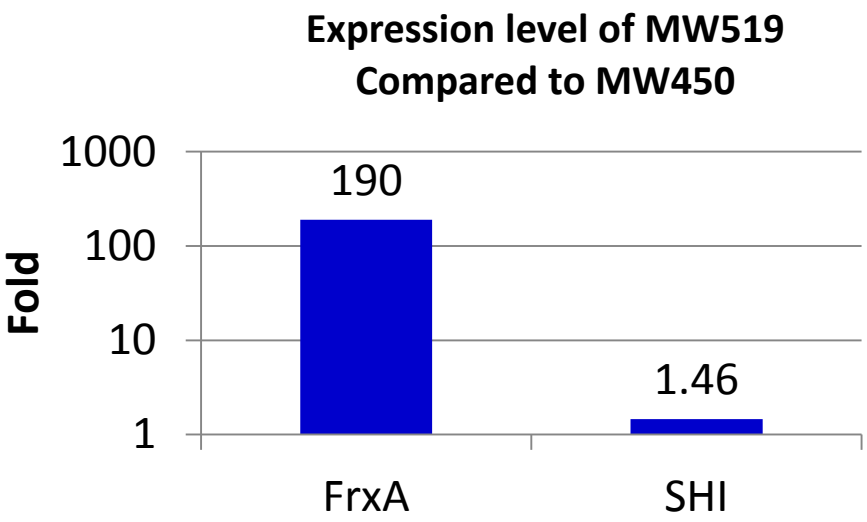
Problem: >30% of the SHI protein lacks the PF0894 catalytic subunit (E3)

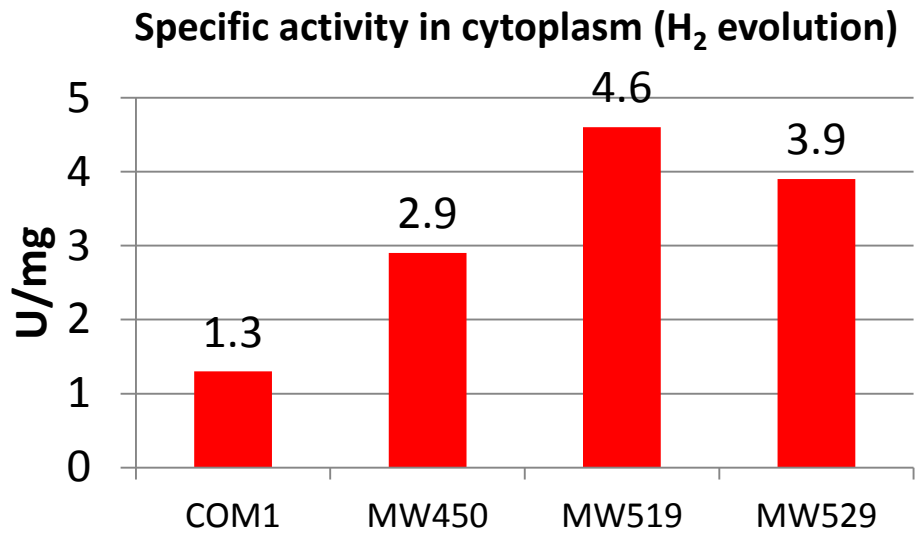
Assumed to be degraded as it lacks the NiFe catalytic site

Goal: to over-express the accessory proteins for SHI maturation to solve the problem

Over-expression of three SHI accessory proteins

Three accessory proteins (FrxA, HypC and HypD) necessary to synthesize the catalytic (NiFe) site of SHI were overexpressed



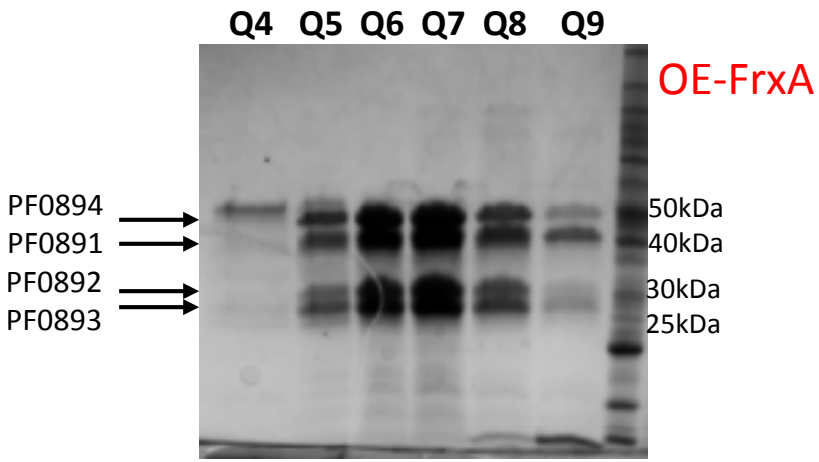


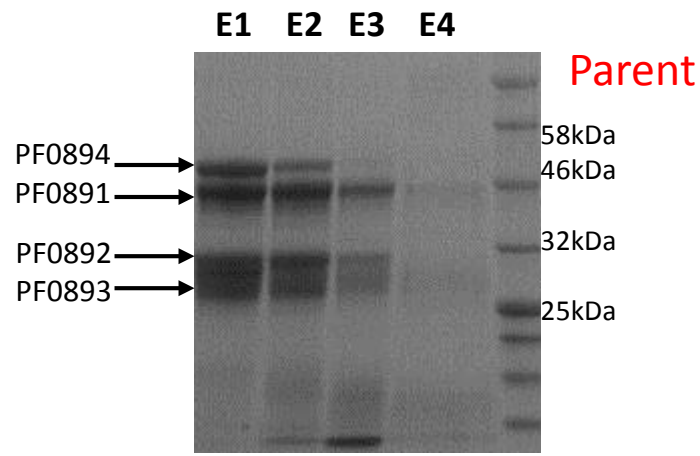
COM1: Original parent strain
MW450: OE 9xHis tagged SHI
MW519: OE 9xHis tagged SHI plus OE-FrxA
MW529: OE 9xHis tagged SHI plus OE-FrxA/OE-HypCD

- Over-expression of *frxA* increased FrxA levels by almost 200-fold (strain MW519)
- **Over-production of FrxA resulted in three-fold higher SHI activity (H_2 evolution)**
- Over-production of HypC/D in the FrxA over-expression strain did not further increase SHI production

SHI purification from cells over-expressing *FrxA*

Step	Total Units* (µmol min^{-1})	Total Protein (mg)	Specific Activity (U/mg)	% Yield	Fold Purification
S100	4328.4	1334	3.24	100	1
Ni-NTA	3041.8	28	108.8	70.3	33.58
Q HP (Q5)	115.8	0.75	155.5	2.7	48
Q HP (Q6-8)	2265.3	14	156	52.3	48
QHP (Q9)	115.9	1.5	77.7	2.7	24



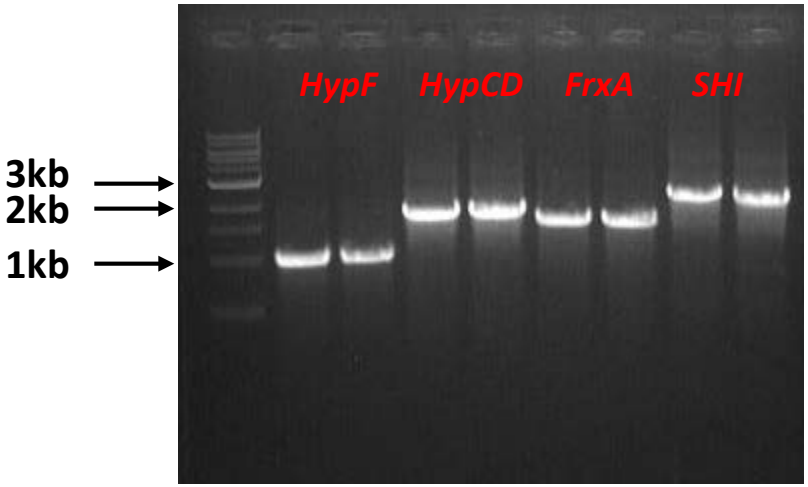


- >30% trimer was observed in previous SHI prep (E3, 20U/mg)
- <10% of total SHI is the trimer in the OE-FrxA strain(MW519) **SHI**
- The yield from a 20L fermentation was low (20g) compared to MW430 (40g)
- **Over expressing *FrxA* dramatically reduces the amount of inactive trimeric SHI**

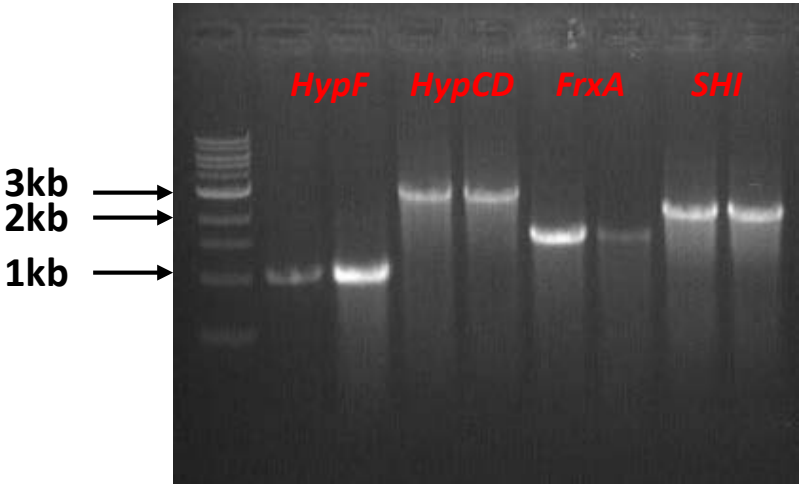
Over-expression of processing protein *HypF*

PCR confirmation of gene loci in strains

MW538



Attempted clone

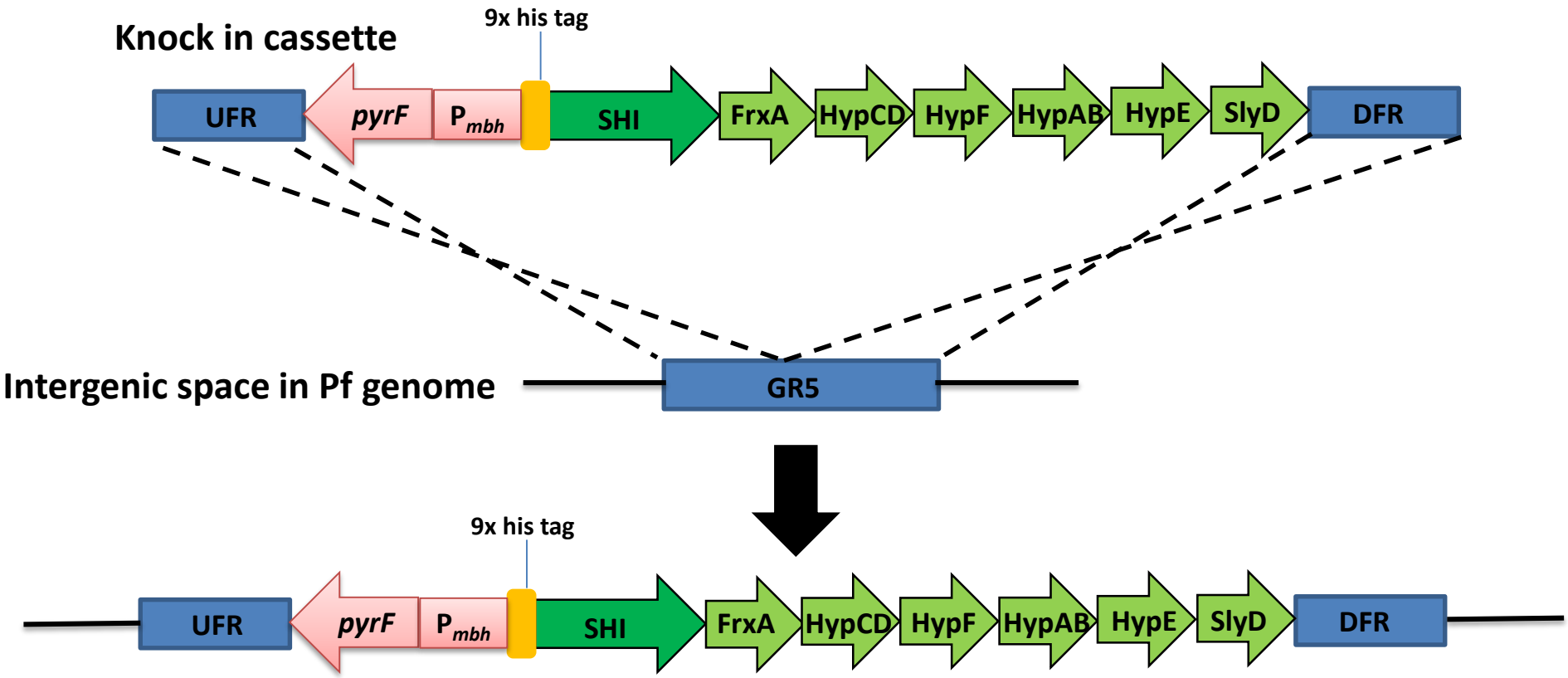


MW538: OE 9xHis tagged SHI plus OE-FrxA/OE-HypCD, along with *pyrF* recycled

- Hundreds of colonies were screened for *HypF* over-expression
- Unable to purify a clean colony of MW538 for *HypF* over-expression, even after 5 rounds of plate purification
- The attempted clone still has *pyrF* selection marker in *HypCD* locus
- Over-expression cassette was not transformed into *HypF* locus
- **HypF cannot be over-expressed without deleterious effects**

New strategy for over-expression of SHI and all 8 maturation genes

All eight maturation genes expressed in addition to a second copy of the SHI genes



- A second copy of SHI and of its 8 maturation genes will be expressed at an intergenic space in the existing OESHI strain
- Genes expression controlled by the P_{mbh} promoter
- **Characterization of the new recombinant strain in progress**

Remaining Challenges and Barriers

- Increase catalytic efficiencies (k_{cat}/k_M) dehydrogenases on biomimics (NMN and NR) to comparative levels of to those on their natural coenzyme (NADP).

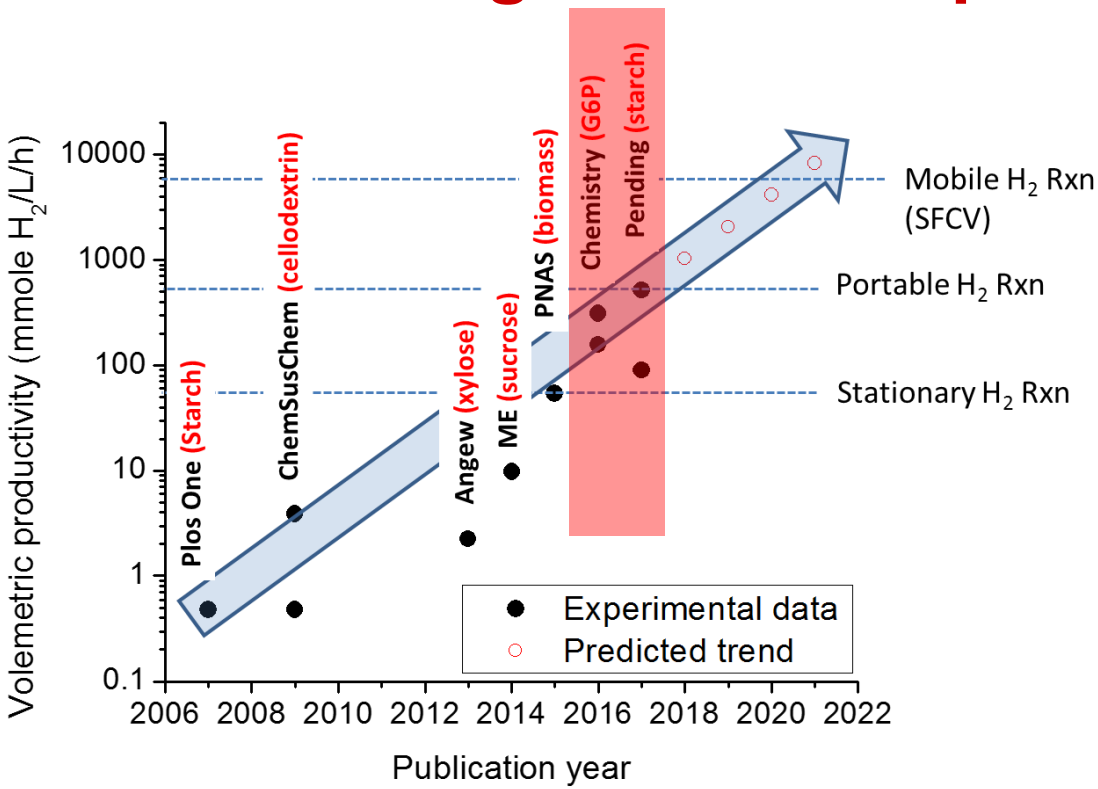
- Stabilize coenzymes for a long time running (partially addressed (via NAD-conjugate) here, future).

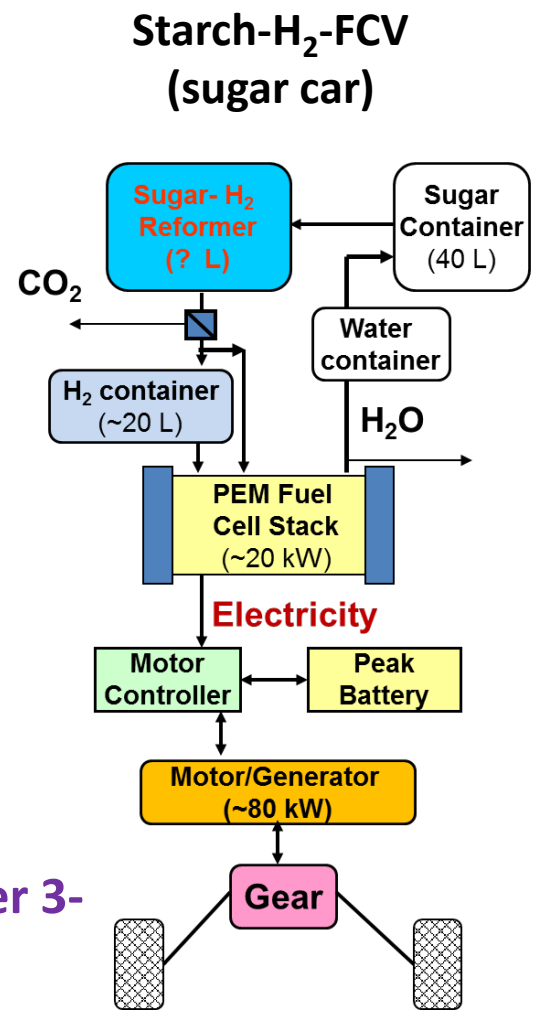
- Decrease SH1 production costs – better expression levels without a decrease in specific activity (i.e., coordinated co-expression of enzyme components, no-cost extension efforts).

- Construct very stable G6PDH-6PGDH-SH1 enzyme complexes featuring electron transport channeling.

- Scale up recombinant enzyme production to kg levels for pilot plant demonstration (need external support)

Increasing volumetric productivity (Rxn)





Zhang's law – Doubling Rxn every year

(increasing 1,000 fold, Rxn = 1 g/L/h)

Goal: On-board H_2 generation @ Rxn = 10 g/L/h (another 3-5 year efforts)

Remaining barriers

1. Concept (in vitro systems are too costly?)
2. Impacts (transportation revolution?)

Zhang. 2009. Is the sugar-powered car science fiction? Energy Environ. Sci. 2:272-282.

Proposed future work (by Dec. 2017)

- **Decrease H_2 production costs by 1000 fold to $10/kg, where production cost will be estimated using H2A model**
  Task 1.2. Replace costly NADP by biomimic – NMN (cont'd)
  Task 1.4. Detailed economic analysis of H_2 production (cont'd)

- **Increase H_2 production rate from 300 to 750 mmole H_2/L/h**
  Task 2.3. Construction of artificial electron transport chains
  Task 2.4. Construction of synthetic metabolons (enzyme complexes)

- **Scale up H_2 reaction volume from 10 mL to 1000 mL**
  Task 3.2. Mass production of hydrogenase (SH1) (UGa)
  Task 3.3. Liter level demonstration (integrated work)

Technology transfer activities

- Virginia Tech and Oak Ridge National laboratory received a US patent US 8,211,681. Biohydrogen production by an artificial enzymatic pathway. (2012).

- We are willing to provide technical help to any entities to commercialize the sugar-to-hydrogen technology and develop the hypothetic sugar-H_2-fuel cell vehicles.

- No special business action taken due to a lack of hands and experienced businessmen.

- (Some Chinese research institutes and companies express strong interests in this project and the in vitro synthetic biology platform because they know enzymes better than US counterparts. China has established the first plant based on this *in vitro* platform for the production of value-added product – myo-inositol.)

Summary

- We achieved the highest biological hydrogen generation rate of 550 mmole H_2/L/h.
- We demonstrated the feasibility of changing coenzyme preference of engineered dehydrogenases from NADP to NAD and biomimics.
- Starch is an off-board H_2 storage compound (i.e., 14.8 H_2 wt. %) and could be a on-board H_2 storage compound.
- We scaled up recombinant *E. coli* enzyme production by 1000-fold and recombinant *P. furiosus* SH1 (hydrogenase) production by 50-fold.

Targets	Units	June 2016 Target	Dec. 2017 Target (estimated)	Year 2020 Target (Plant gate)
Production cost	\$/kg H_2	1000	10	10 (year 2020)
Productivity	mmole H_2/L/h	550 (achieved)	750	2,000
Reactor volume	L of reactor	1 (to be finished)	1	65,000*
				*1,500 kg H_2 per day

Technical Back-Up Slides

Appraisal of enzymes as biocatalysts

Basic facts

- ❑ Most enzymes are proteins
- ❑ Biocatalysis catalyzed by enzymes has highly chemical selectivity (no side product)
- ❑ Most enzymes work at mild reaction conditions (low temperature, 1 atm, neutral pH, and aqueous phase)
- ❑ Enzymes do not require costly precise metals

Conflicting concepts (academic researchers versus industrial enzyme experts)

× For academic researchers, enzymes are VERY costly (e.g., **billion dollars per kg**)
√ For industrial enzyme experts, bulk enzymes are less costly (e.g., **10 dollars per kg**)

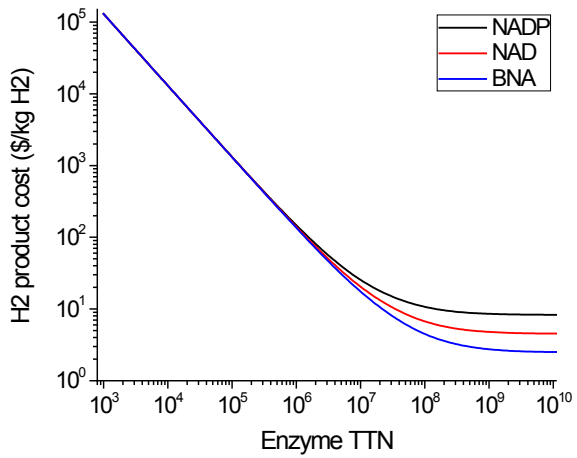
× For academic researchers, enzymes are very labile, losing activities within hours or days
√ For industrial enzyme experts, some enzymes (e.g., immobilized or engineered) are very stable, lasting months and years (e.g., glucose isomerase for HFCS production, protease in detergent, glucose dehydrogenase in blood sugar test strips).

× For academic researchers, enzymes have narrow reaction conditions in terms of pH, temperature and solutions.
√ For industrial enzyme experts, some enzymes (engineered or discovered from extremophiles) can work on a large temperature range from 0 – 100°C, pH from 1 to 14, from aqueous solution to 100% organic solvent

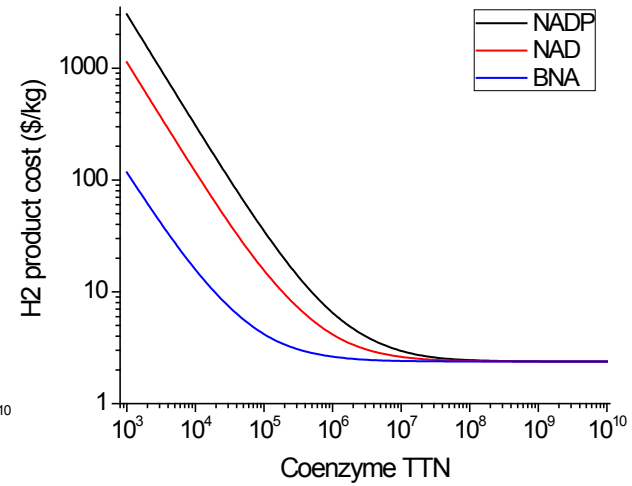
Decreased H_2 production costs

Quantitative indicator of enzymes and coenzyme: **total turn-over number (TTN)**, mole product/mole enzyme

Effects of enzyme stability



Effects of coenzyme stability



Preconditions for low-cost hydrogen production

- ❑ **Low cost enzyme production ($10-20/kg)**
- ❑ **High stability of enzymes (TTN = 10^{8-9} mole product/mole enzyme)**
- ❑ **High stability of coenzymes (TTN = 10^{6-7} mole product/mole enzyme)**
- ❑ **Low-cost of coenzymes ($100/kg)**

Key directions: (1) discovery of better enzymes (Task 1.3), (2) engineering of dehydrogenases on biomimetic cofactors (Task 1.2), and (3) mass production of enzymes (Tasks 3.1 & 3.2).

Zhang, *Biotechnol. Adv.* 33, 1467–1483 (2015).

Enzyme stability

EC	Enzyme name	*Source*	Form	Cond.	TTN
1.1.1.44	6-phosphogluconate hydrogenase	*T. maritima*	Free	80°C	2.4×10^8
2.2.1.2	Transaldolase	*T. maritima*	Free	60°C	1.7×10^7
3.1.3.11	Fructose 1,6-Bisphosphatase	*T. maritima*	Free	60°C	2×10^7
5.4.2.2	Phosphogluomutase	*C. thermocellum*	Free	60°C	7.1×10^7
5.3.1.5	Xylose (glucose) isomerase		Immobilized	50-60°C	5.0×10^8
5.3.1.6	Ribose-5-phosphate isomerase	*T. maritima*	Free	60-70°C	2.2×10^8
5.3.1.9	Phosphoglucose isomerase	*C. thermocellum*	Free Immob.	60°C	3.2×10^7 1.1×10^9

Our goal:
1 kg of enzyme produces 300 kg of H2 @ TTN = 4 E7 ➔ \$0.10/kg H_2