Innovative, Scalable and Sustainable Bio-manufacturing Routes to Precursors of the High Explosive Hexanitrohexaazaisowurtzitane (CL-20)

WP20-1110

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14. ABSTRACT
The technical objectives of this project are listed below:
- Identify, construct and optimize novel biosynthetic routes towards benzylamine
- Employ strain engineering techniques to improve strain performance and/or eliminate unwanted activities in *E. coli*
- Utilize Halomonas as a novel production host with improved fermentative properties

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Project Team

- University of Manchester
  - Prof. Nigel Scrutton
  - Dr Rosalind Le-Feuvre
  - Dr Jonathan Wilkes

- Naval Air Warfare Center Weapons Division, China Lake
  - Dr Benjamin Harvey

Manchester – NAWCWD Partnership

High Performance Synthetic Fuels

- Coupling of biological precursor supply to chemistry of production of energy dense fuels / materials
- Demonstration of performance, evaluation and accreditation of new fuels
- Scaling of production, uniting biology, chemistry and engineering e.g. distributed manufacture in theatre

Seawater fermentation

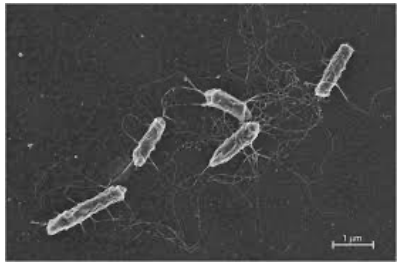
A game changer for distributed biomanufacturing at scale

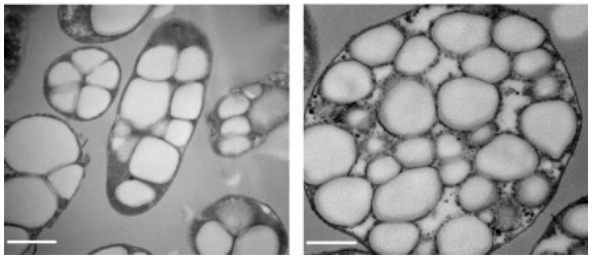
- *Halomonas* is a salt-tolerant bacteria
- Biomanufacturing in non-sterile seawater.
- Low CAPEX/OPEX burden.
- Enables distributed biomanufacturing

From seawater to jet fuel: NAWCWD teams up with ONR Global for biosynthetic research

Published: Mar 11, 2019

NAVAL AIR WARFARE CENTER WEAPONS DIVISION, CHINA LAKE, Calif. – "There are plenty of examples how biology has made superior materials," said Dr. Patrick Rose, science director for Synthetic Biology at the Office of Naval Research Global. "For example, spider silk for its elasticity or nacre in sea shells for its strength and toughness. Synthetic biology is a technology platform that allows scientists to apply engineering principles to re-engineer biological processes and make desired products from such amazing natural features."





Halomonas sp. used to biomanufacture bioplastics (PHA & PHB) on the kilotonne scale.

WP20-1110
Innovative, Scalable and Sustainable Bio-manufacturing Routes to Precursors of the High Explosive CL-20

Performers: Prof Nigel Scrutton, Dr Benjamin Harvey, Dr Jonathan Wilkes, Dr Ros Le-Feurve

Technology Focus

- Develop scalable and environmentally sustainable bio-manufacturing routes to benzylamine.

Research Objectives

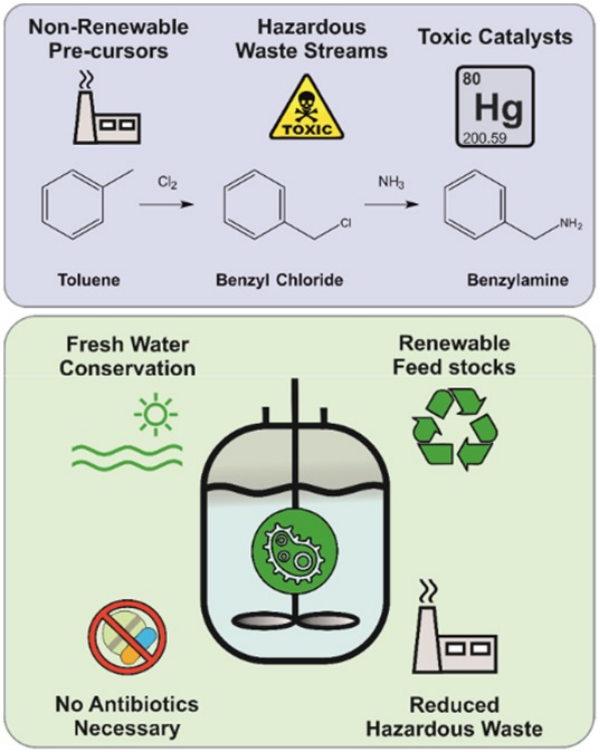
- Construct stable, biosynthetic benzylamine-producing DNA constructs initially within *E. coli* and then within *Halomonas*.

Project Progress and Results

- Identified additional avenues to increase benzylamine titres from glucose, glycerol or other waste feedstocks.
- Identified gene deletion targets in *E. coli* that may eliminate unwanted activities.

Technology Transition

- Follow-on SERDP effort for the continued development of robust microbial production platforms.
- Partnerships with C3 BIOTECH, Future Biomanufacturing Research Hub and NAWCWD.



Background

- Project Initiated - Spring 2020
- WPSON-20-C2: Advanced Synthesis Techniques for Military-Relevant Energetic Materials or Significant Precursors
- Develop a sustainable synthetic biology approach to the manufacture of benzylamine, a precursor of the high explosive CL-20

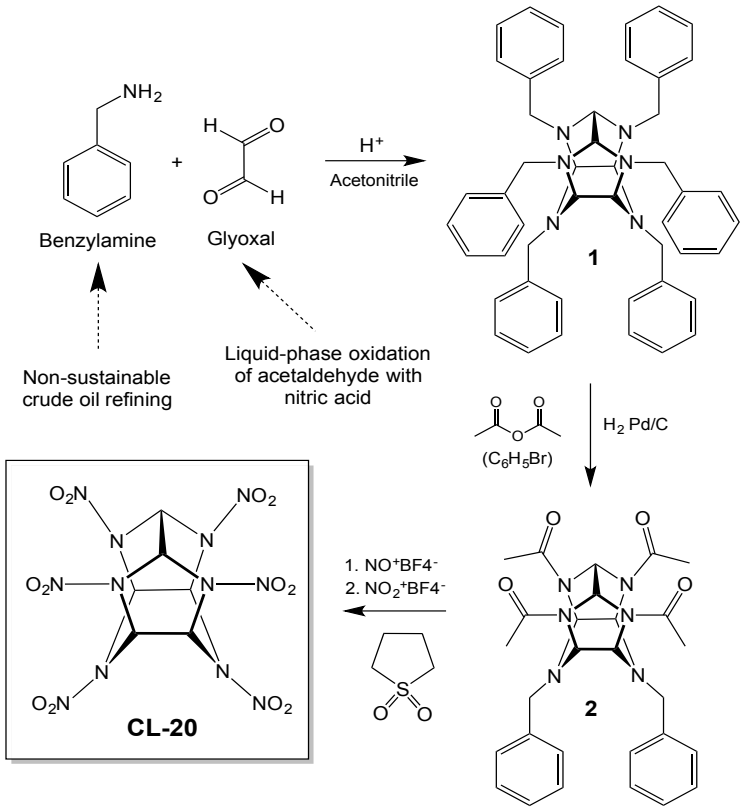
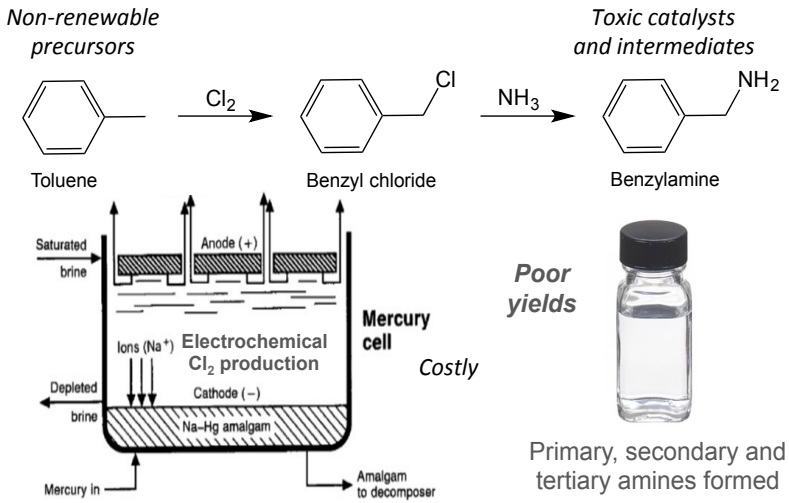


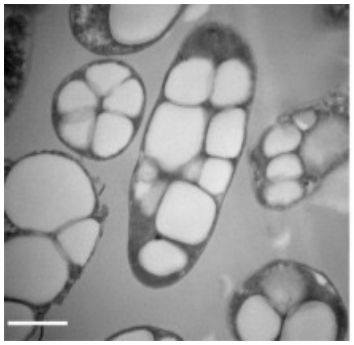
Figure 1. Synthetic route of CL-20.

Current state of the art

Chemical manufacture



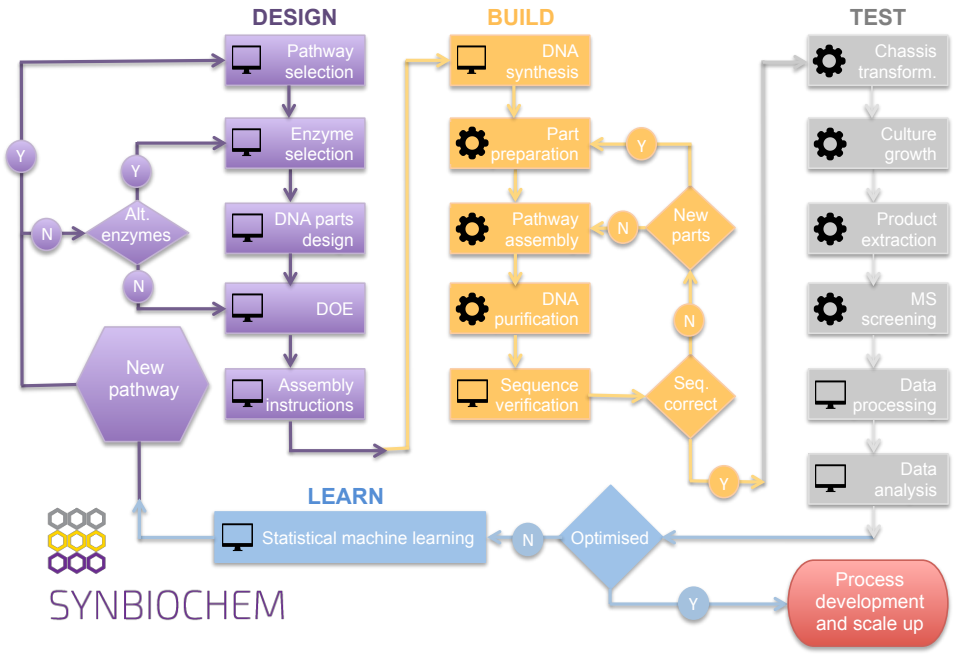
Biological manufacture?



- Precursors – petrochemical derived and non sustainable
- Benzylamine made from toluene derived from crude oil
- Uses mercury cell for chlorination to make benzyl chloride; expensive to buy, operate and highly toxic
- Benzyl chloride the reacts with ammonia; poor yielding reaction
- Overall prohibitive cost for CL20 manufacture

Technical Objectives

- Identify, construct and optimize novel biosynthetic routes towards benzylamine
- Employ strain engineering techniques to improve strain performance and/or eliminate unwanted activities in *E. coli*
- Utilize *Halomonas* as a novel production host with improved fermentative properties



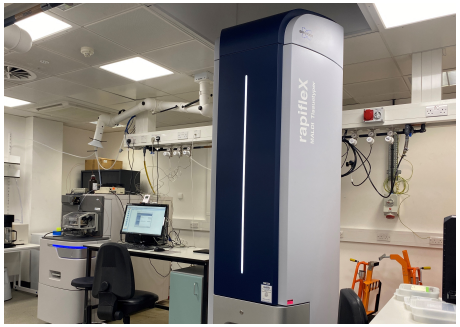
Delivery vehicle: SYNBIOCHEM automated HTP platform for microbial strain engineering

Technical Approach

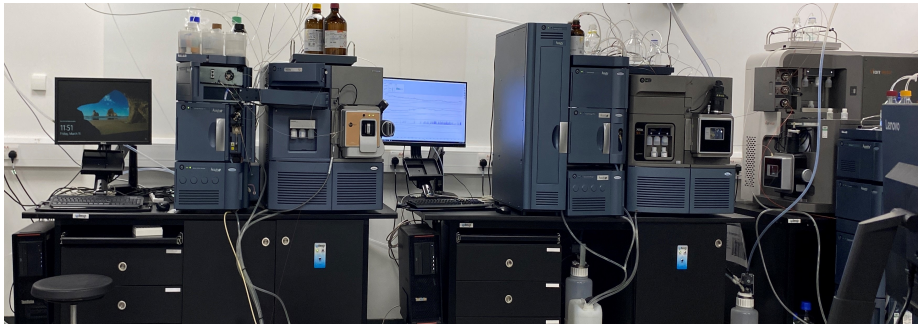
Iterative HTP Strain Engineering

Bio-part selection => Pathway assembly => Pathway performance =>
Host engineering => Host optimization => Productivity evaluation & improvement

High Throughput Robotics



Mass Spectrometry



Biotechnology and Biological Sciences Research Council

Engineering and Physical Sciences Research Council

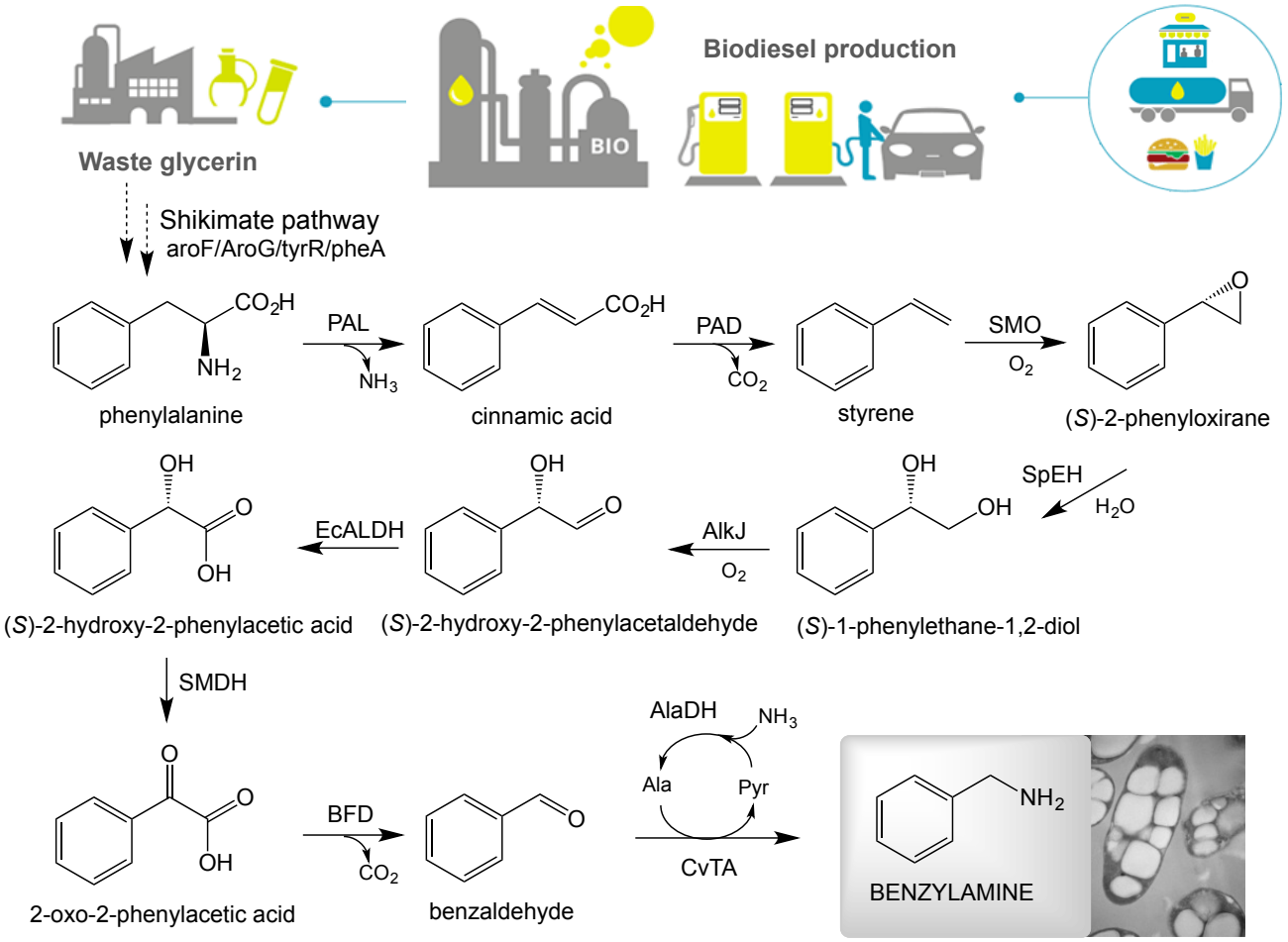
Timeline and Deliverables

		Year 1				Year 2			
	Milestones	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
M1	Prototypical *E. coli* strain for benzylamine production								
M2	Combinatorial optimisation of the *E. coli* pathway								
M3	LEARN parameters to guide *Halomonas* engineering								
M4	Prototypical *Halomonas* strain producing high-titres of phenylalanine								
M5	High-titre benzylamine pathway engineered and operative in *Halomonas*								
M6	Production of benzylamine from *Halomonas*								
M7	Testing of the isolated product in China Lake								
	Deliverables								
D1	Combinatorial library of benzylamine production strains in *E. coli*								
D2	Selected high titre phenylalanine production strain in *Halomonas*								
D3	Selected high titre benzylamine production strains in *Halomonas*								
D4	Biomanufactured samples of benzylamine available for CL-20 synthesis								

Funded as a 1 year limited scope project

- Milestones M1-6 accomplished
- Deliverables for Y1 achieved
- Samples for chemical synthesis require DSP/work up (subject of follow on request)
- Program partially affected by SARS Covid-19 (laboratory closure)

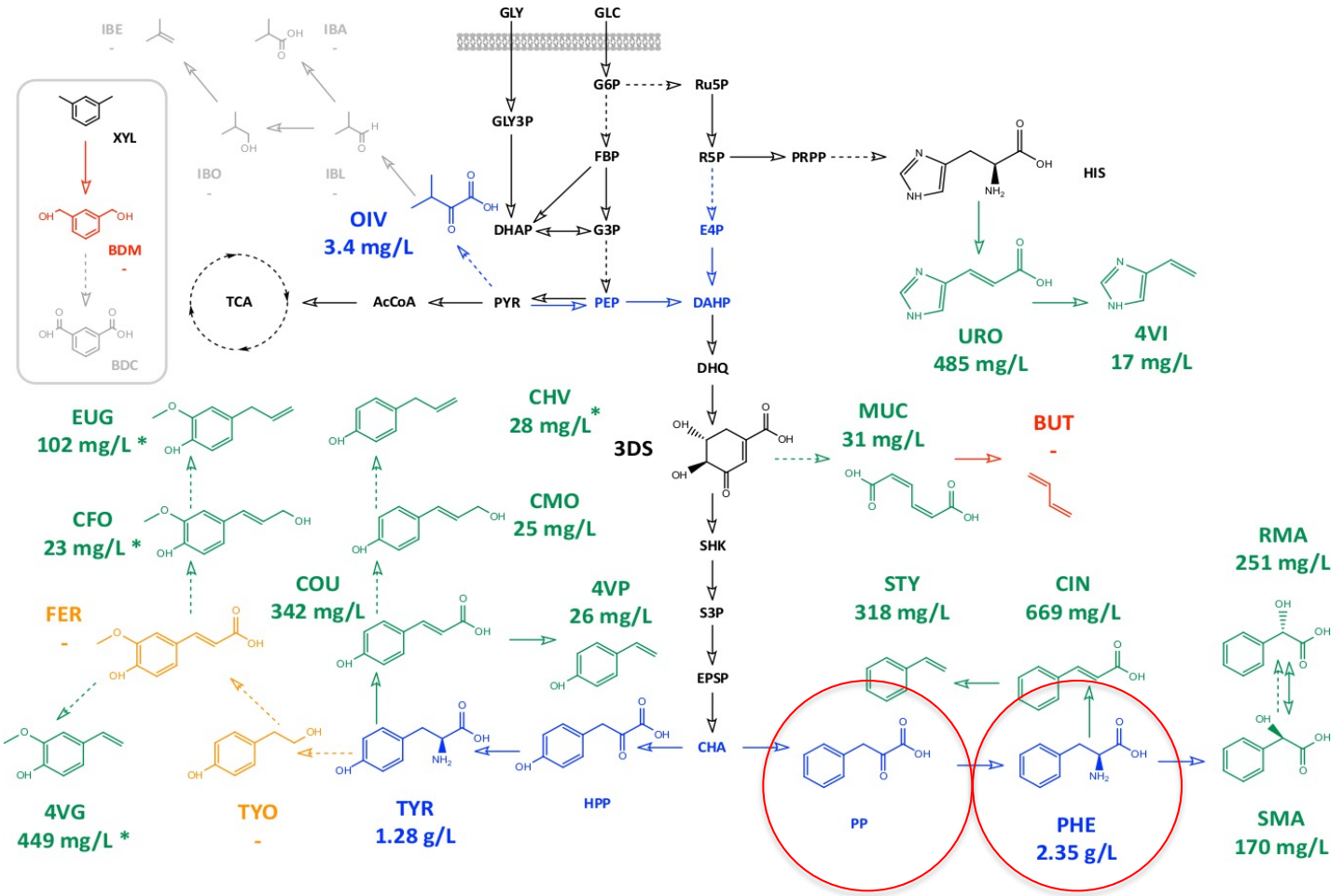
Nine-step prototype pathway



SYNBIOCHEM HTP capability

SYNBIOCHEM 'stress test' for materials monomers => elevated phenylamine production.

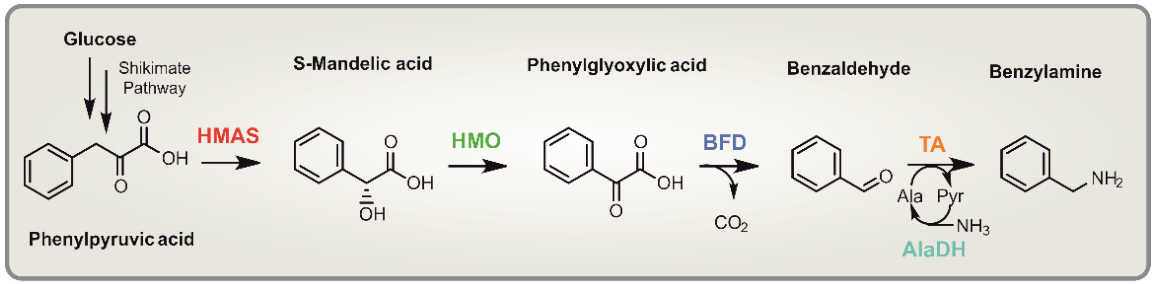
- Out of 24 targets: 17 material monomers produced combining >150 genetic parts into 150 unique biosynthetic pathways assembled and evaluated in under 3 months.

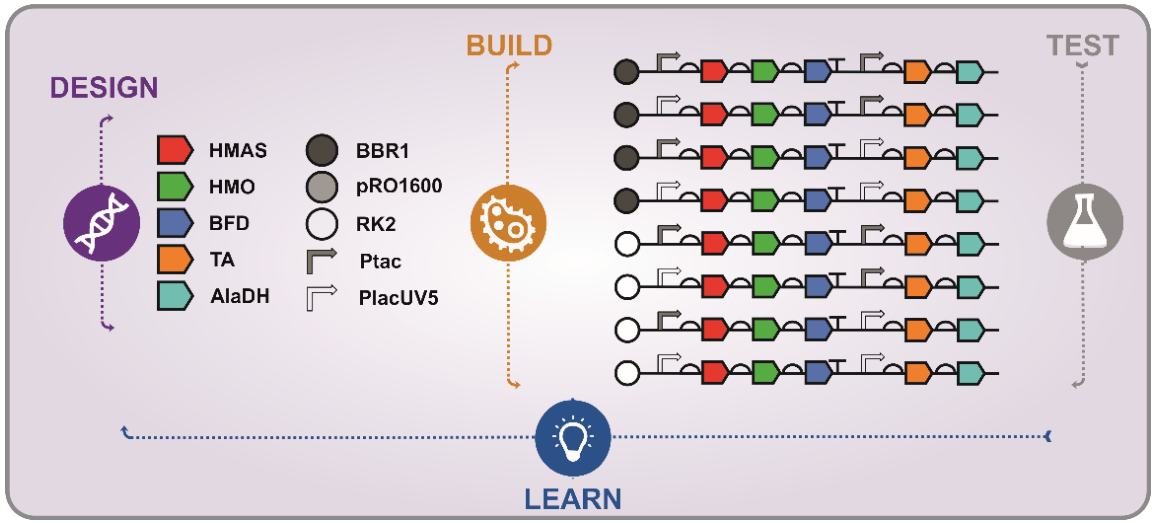


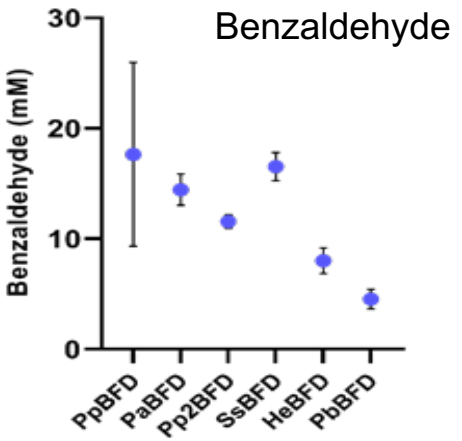
Metabolic Engineering 60, 168 (2020)

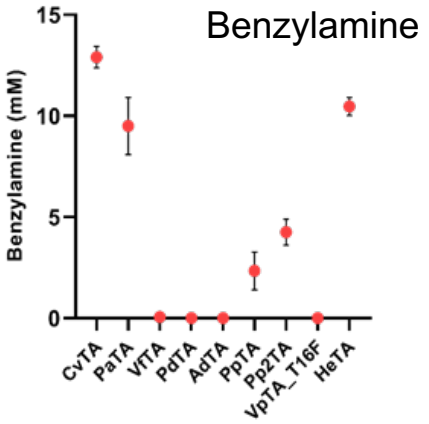
Results – New Routes to Benzylamine

Combinatorial exploration of bio-part functionality in pathways





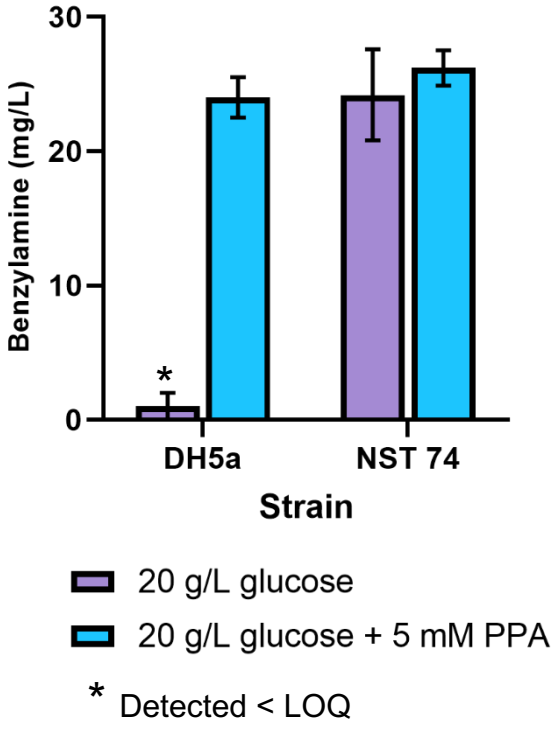




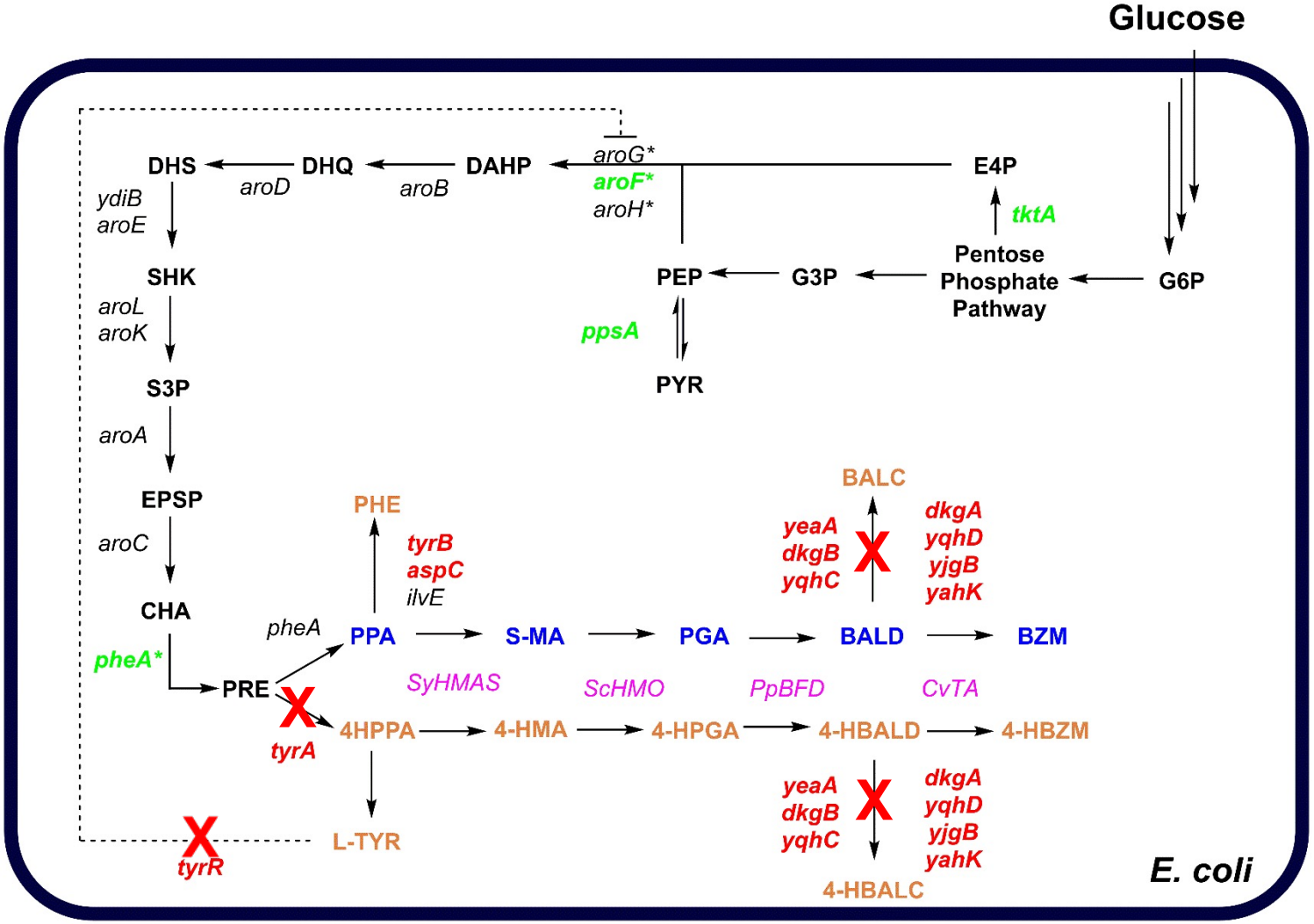
Design: Minimize reaction steps; multiple parts
Build: Use base strains; biocatalyst selection
Test: Biotransformations; mass spectrometry

Results – Evaluation of Pathway Performance

- Biosynthetic pathway (p434T2 BZM 1P) was tested in *E. coli* DH5α, (25 mL shake-flask, M9 minimal media + 20 g/L glucose)
  - Benzylamine produced < LOQ in absence of supplementation
  - 24.0 ± 1.5 mg/L benzylamine produced with 5 mM phenylpyruvate (PPA) supplementation
- Production using phenylalanine overproduction strain *E. coli* NST 74 (25 mL shake-flask, M9 minimal media + 20 g/L glucose)
  - 24.2 ± 3.4 mg/L benzylamine produced after 48 h
  - 26.2 ± 1.3 mg/L benzylamine produced with 5 mM PPA supplementation
- LC-MS analysis also revealed accumulation of:
  - Phenylalanine (~1 g/L)
  - Benzyl alcohol (~80 mg/L)
  - Hydroxy-benzyl alcohol (~150 mg/L)



Results – Evaluation of Pathway Performance

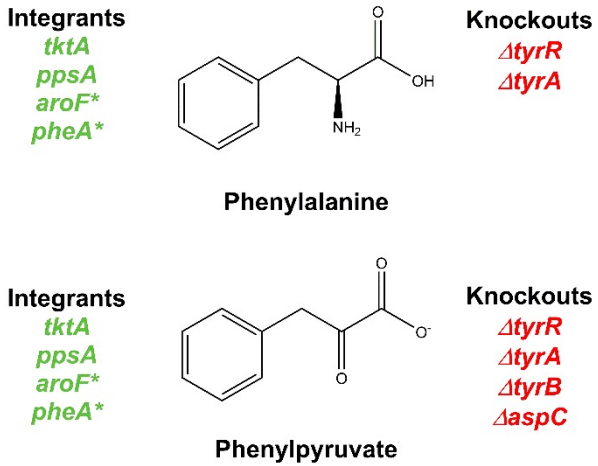


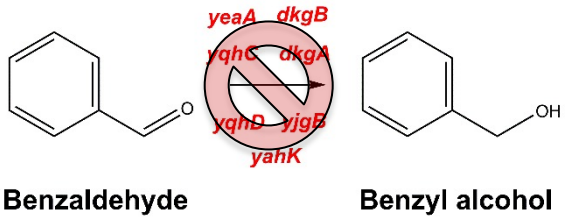
Results – Strain Engineering for Improved Performance (*E. coli*)

Genome editing using CRISPR-Cas12a has been employed to develop new strains with improved phenotypes:

- Phenylpyruvate (PPA) overproduction:
  - tyrR & tyrA KO (**DH5α ΔtyrR ΔtyrA**) ✓
  - Genes for overproduction integrated into native LacZ gene (**DH5α Phe+ ΔtyrR ΔtyrA**) ✓
  - tyrB KO (**DH5α Phe+ ΔtyrR ΔtyrA ΔtyrB**) ✓
  - aspC KO ongoing…

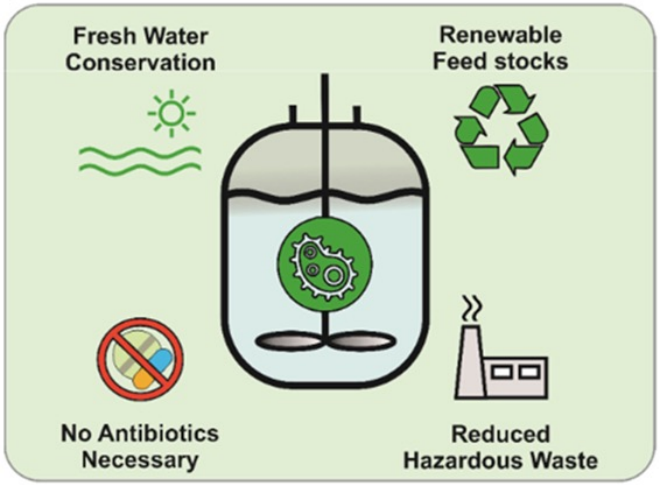
- Eliminate benzaldehyde interception:
  - *E. coli* MG1655 **R**educed Aromatic **A**ldehyde **Re**duction (RARE) strain (Kunjapur et al. 2014) demonstrates improved bioproduction of benzaldehyde
  - Currently investigating these KO in PHE/PPA overproduction strains





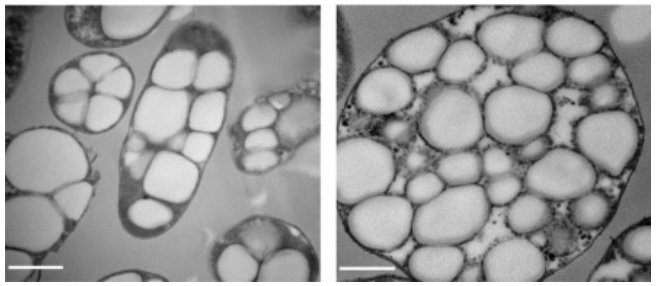
Results – Transitioning to *Halomonas*

- *Halomonas* produces 2.5 mg/L benzylamine with 5 mM PPA supplementation



- Observations from *E. coli* strain engineering have/are being used to guide further *Halomonas* strain development
  - Successful KO of tyrR, trpR
  - Random mutagenesis & toxic analog selection used to create mutant PHE overproduction library, library screening for highest performing variant ongoing.

- *Halomonas* readily utilizes PHE as a carbon/nitrogen source
  - Exploring engineering and fermentation strategies to prevent this



- Benzylamine is a water-soluble base
  - Difficult to extract from aqueous fermentation mixtures
  - High pH, high salinity preferred by *Halomonas* will aid in the isolation of benzylamine from the culture broth via liquid-liquid extraction (LLE)

Next Steps

- Optimize builds for production pathways in *E. coli* (on going) – driving increased yields/titres
- Integrate into the host genome (or essential metabolic plasmids) for improved process stability – avoid use of antibiotic selection markers
- *Halomonas* production strain for improvements– non sterile bioproduction (if required); improved titres
- Methods for fermentative production and DSP of benzylamine using both *E. coli* & *Halomonas* – process scaling technology development (Phase 2 proposal)

Technology Transfer

- **Confidentiality:** intention is to file patents on relevant aspects of the project (pathways & processes).

- **Scale up/de-risk:** C3 BIOTECH - expert in large scale fuels / chemicals production in *E. coli* and *Halomonas.*

- **Partnerships:** Future Biomanufacturing Research Hub (Manchester) and NAWCWD.

- **Large scale:** US Army (Combat Capabilities Development Command – Chemical Biological Center).

Key Points

- We have addressed the original 'Statement of Need' WPSON-20-C2 - producing biosynthetic benzylamine, a precursor to CL-20
- Continued pathway and strain engineering established to improve product titers and strain process stability
- Large-scale fermentation and DSP methods now required to enable scale-up
- Ambition: Flexible large-scale production platforms based on *E. coli* and/or *Halomonas*

Immediate Future Research

- Results to date demonstrate that the bio-production of benzylamine is feasible in microbial cell factories

- To further address the WPSON-20-C2 ‘Statement of Need’ the development of robust fermentation and DSP methods for the scaling and recovery of bio-benzylamine is necessary

- This can be complemented by the continued development of both *E. coli* & *Halomonas* as chassis for improvements in titers and process adaptability

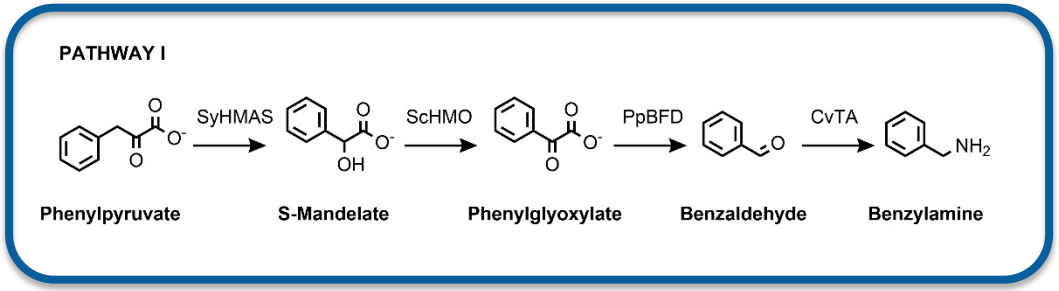
Future Research

- Phase 2 Proposal:
  - Additional 12 months
  - Approximate investment $250,000

- WP1: Downstream Processing Trials
  - Solvent trials for LLE
  - pH & salt trials
  - Distillate quality trials
  - Distillation optimization

- WP2: Investigation of Batch & Continuous Modes
  - Scoping batch and continuous fermentation conditions
  - Optimization of media composition / fermentation conditions
  - Provision of bio-benzylamine for CL-20 synthesis

Results – Identification of Other Biosynthetic Routes



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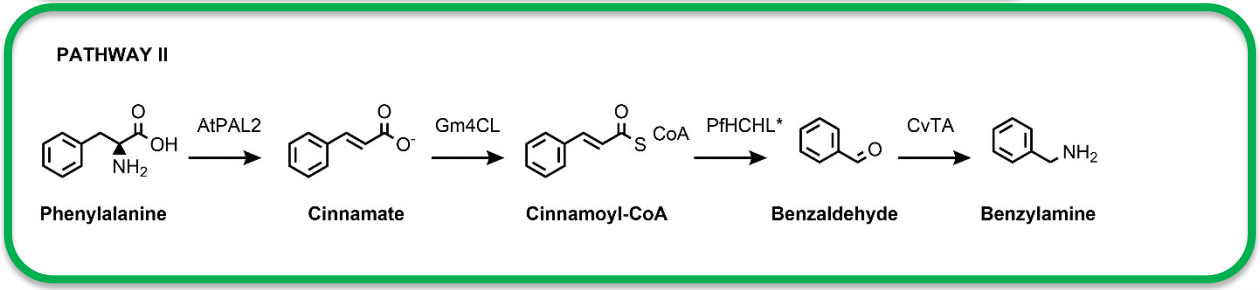
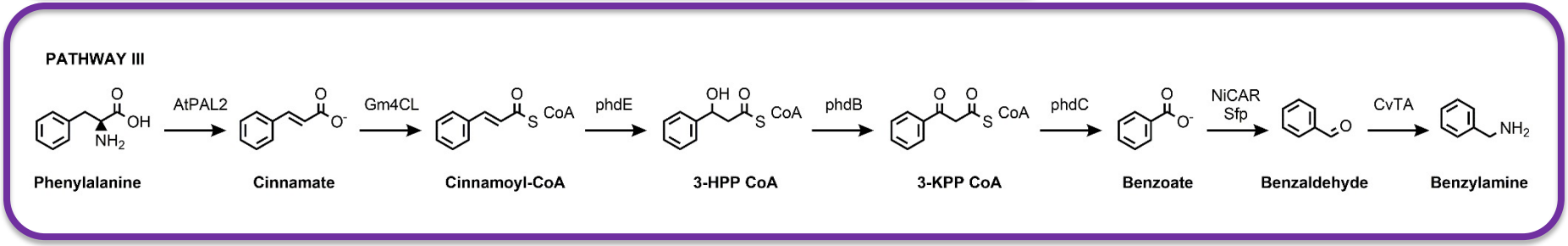
Assembled ✓
Tested ✓



* Directed Evolution required to change selectivity

Designed ✓
Assembled ✓
PfHCHL screening…



Designed ✓
Assembly ✓
screening…

Thank you

- University of Manchester
  - Prof. Nigel Scrutton
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