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LABORATORY
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Engineering β -ketoadipic Acid Production in *Pseudomonas putida* KT2440

Cooperative Research and Development Final
Report

CRADA Number: CRD-21-18379

NLR Technical Contacts: Robert Baldwin and Gregg
Beckham

The National Laboratory of the Rockies is a national laboratory of the U.S. Department of Energy, Office of Critical Minerals and Energy Innovation, operated under Contract No. DE-AC36-08GO28308.

Technical Report
NLR/TP-2A00-98487
January 2026

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Cooperative Research and Development Final Report

Report Date: December 16, 2025

In accordance with requirements set forth in the terms of the CRADA agreement, this document is the CRADA final report, including a list of subject inventions, to be forwarded to the DOE Office of Scientific and Technical Information as part of the commitment to the public to demonstrate results of federally funded research.

Parties to the Agreement: Triad National Security, LLC and Battelle Memorial Institute, and Technology Holding, LLC

CRADA Number: CRD-21-18379

CRADA Title: Engineering β -ketoacidic Acid Production in *Pseudomonas putida* KT2440

Period of Performance: 8/15/2022 – 9/30/2025

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Sponsoring DOE Program Office(s):

Office of Energy Efficiency and Renewable Energy (EERE), Bioenergy Technologies Office

Joint Work Statement Funding Table showing DOE Commitment:

Estimated Costs	Project Year 1	Project Year 2	Totals
NLR	\$370,000.00	\$370,000.00	\$740,000.00
LANL	\$180,000.00	\$180,000.00	\$360,000.00
PNNL	\$170,000.00	\$170,000.00	\$340,000.00
Total Government	\$720,000.00	\$720,000.00	\$1,440,000.00

Executive Summary of CRADA Work:

We propose a 2-year project to enable commercialization of an Agile Biofoundry (ABF) related, performance-advantaged bioproduct, β -ketoadipic acid (β KA), in an ABF-relevant, industrially-relevant host, *Pseudomonas putida* KT2440. Informed by techno-economic analysis (TEA), our β KA production goals are to enable a 40 g/L titer, 0.5 g/L/h productivity, and a 40% molar yield from hydrolysate sugars at the 1 kg scale. This material will be used to produce β KA-nylons.

CRADA benefit to DOE, Participant, and US Taxpayer:

- Enhances the laboratory's core competencies

Summary of Research Results:

Task 1: Generation of non-intuitive targets for improved β KA production.

Discussion: We will leverage a baseline strain able to consume glucose and xylose and produce β KA. This strain will form the initial chassis for β KA production in the proposed project. From this baseline strain, we will pursue two parallel approaches to identify non-intuitive targets to improve β KA rates and titers. First, we will construct a randomly barcoded transposon sequencing (Rb-Tn-Seq) library into the above strain. An existing plasmid-based, fluorescence biosensor with a dynamic range tuned to detect at high millimolar concentrations will be transformed into this library. Coupling the fluorescence-based (GFP) biosensor with the Rb-Tn-Seq library will enable enrichment by single cell sorting and sequencing of strains capable of improved β KA production based on the union of positive fitness-based enrichment in the Rb-Tn-Seq library and increased GFP fluorescence. This may require method development in terms of tuning the biosensor range and cultivation conditions to achieve sufficient differential resolution. Sequencing of the enriched library will be conducted by a commercial vendor. Genes that, when disrupted, result in greater production of β KA (based on fluorescence) will be knockout and knockdown targets. Genes that result in less production of β KA (based on fluorescence) when disrupted will be overexpression targets.

As a complementary approach, we develop and leverage of another method, overexpression Tn-Seq (OERb-Tn-Seq). Here, we will construct a second Rb-Tn-Seq library in which the transposon used to construct the library contains the tac promoter at one end. All beneficial non-intuitive targets identified by Rb-TnSeq and OE-Rb-Tn-Seq will be evaluated individually by reverse engineering into the baseline strain.

The second approach will rely on adaptive laboratory evolution (ALE) in the base strain with the β KA catabolic genes, *pcaIJ*, re-integrated back into the strain. Mutant(s) that exhibit an improved growth rate relative to the unevolved parent will be submitted to FACS, in order to isolate cells with increased product titers. Full genome resequencing will be performed on the isolates, and potentially causative mutations will be identified. Potentially beneficial mutations will be evaluated individually by reverse engineering into the base strain.

Improved strains will be evaluated in 0.5-L bioreactors. We will utilize the number of feeding pulses (using a DO-stat fed-batch mode) as a proxy for monitoring sugar utilization rates and β KA productivity. In addition, samples will be collected during the cultivation and end-time point for high performance liquid chromatography (HPLC) analysis. Mutations generated by either method that are found to improve production of β KA will be evaluated in combination to determine if they are redundant, additive, or synergistic and, ultimately, identify an optimal combination of mutations that improve β KA production. The batches of β KA produced during these phases of the project (~10-100 grams likely to result from these works) will be sent to Technology Holding for separation, purification, and small-scale materials testing.

Contractors' Deliverables: At least two libraries in a β -ketoacid (β KA) producing *P. putida* strain. The libraries alters every gene in the *P. putida* strain (NLR). Phenotype-based library screens using designed biosensor (LANL). Identification of gene targets correlating to improved performance (NLR and LANL). Gene validation in the base strain (NLR and LANL). Bioreactor studies of top performing strains (NLR). Top performing strain sent to PNNL (NLR). A *P. putida* strain with a combination of non-intuitive mutations that improve β KA production (NLR).

Participant's Deliverables: Participation in discussion of experimental planning and outcomes.

Task 1 Results: The work completed and outcomes of this task are covered by the following report:

<https://pubs.rsc.org/en/content/articlehtml/2025/gc/d5gc01813g>

Task 2: Global metabolomics of new strains.

Discussion: At the end of each Design-Build-Test-Learn (DBTL) cycle from Task 1, the project team will engage with ABF partner PNNL to conduct global metabolomics of the optimized strain, relative to the base strain. This analysis will provide insights especially for the identification of competing pathways that can be deleted from the strain, as well as to identify metabolites that accumulate during biosynthesis of β KA. We have substantial experience in the ABF conducting metabolomics in *P. putida* related to the shikimate pathway, such that detailed protocols and analysis pipelines for this work exist already and are well established in the team.

Contractors' Deliverables: Global metabolomics analysis on intracellular and extracellular metabolites on the selected strains plus global proteomics analysis (NLR and PNNL)

Task 2 Results:

PNNL analyzed β KA-producing strains (GR038 and GR048) under three experimental conditions: glucose, xylose, and a combination of glucose and xylose. Through global proteomics analysis, 778 proteins were identified as differentially expressed in at least one of these conditions. Notably, significant overlap in differentially expressed proteins was observed between the glucose-only and glucose+xylose conditions. Furthermore, protein-level changes were more pronounced under glucose-grown conditions compared to xylose-grown conditions.

In parallel, global metabolomics analysis focused on intracellular metabolites revealed elevated concentrations of several metabolites, including numerous amino acids, in strain GR038 under glucose-fed conditions. Analysis of extracellular metabolites highlighted a notable accumulation of intermediates in the glycolysis pathway, suggesting inefficient glucose utilization in strain GR048. These findings provide critical insights into metabolic and proteomic responses, as well as the underlying inefficiencies in glucose metabolism in strain GR048. Some additional details of this work and the resulting outcomes are provided in the accompanying report:

<https://pubs.rsc.org/en/content/articlehtml/2025/gc/d5gc01813g>

Task 3: Process integration and scale-up for TRY goals.

Discussion: At the end of each DBTL cycle, we propose to conduct bioreactor cultivations in pH-controlled 10-L bioreactors to benchmark the newly engineered, stacked strains relative to the original base strain. This will involve at least two primary bioreactor campaigns during the project that could produce (potentially) up to ~1 kg of material at a time when run in triplicate, each batch of which will be delivered to Technology Holding. At the end of the project, we will conduct 10-L bioreactor cultivations in triplicate to be able to produce > 1 kg of β KA to deliver to Technology Holding for separation, purification, and polymerization into β KA-nylons.

Task 3 Results: The work completed and outcomes of this task are covered by this report:

<https://pubs.rsc.org/en/content/articlehtml/2025/gc/d5gc01813g>

Final Deliverable from all Parties is a report addressing objectives and deliverables in accordance with Article X. Contractors will deliver report to OSTI.

This report serves to meet the requirement for the CRADA Final Report with preparation and submission in accordance with the agreement's Article X.

Subject Inventions Listing: None.

ROI#: None.