Muconic Acid Production from *P. putida* using High Protein Algae Hydrolysate

Ryan Spiller, Eric P. Knoshaug, Nick Nagle, Tao Dong, Bonnie Panczak, Stefanie VanWychen, and Jake Kruger

Abstract

The composition of algal biomass is highly dynamic, with protein, lipid, and carbohydrate contents varying in response to nutrient and environmental conditions during cultivation. Because shifts in biomass composition are often associated with reduced biomass productivity, production costs can often increase if targeting higher biomass compositional quality (enriched in carbohydrates or lipids at reduced protein content) as input for the algal biorefinery. The optimal algal biorefinery configuration is thus a function of many factors. One of the key strengths of the Combined Algal Processing (CAP) process is the versatility of feedstocks and products produced. The concept has been demonstrated with ethanol and a variety of carboxylic acids (succinic, butyric, muconic) as coproducts along with lipid upgrading to biofuel. Modification of the approaches, processes and downstream upgrading to fuels has allowed the CAP process to reduce costs and improve efficiency. Muconic acid is a high-value, potential fermentation coproduct of interest because it can be easily converted to adipic acid, a high-volume monomer for the production of nylon and other valuable consumer plastics. As such, the production of muconic acid through CAP was explored to expand the suite of products from algal biomass and to begin exploring the valorization of high protein content biomass from rapidly grown algae biomass. We have established initial performance parameters and shown that the range of substrates consumed by the muconic acid-producing microbe, *Pseudomonas putida*, includes at least glucose, mannose, glycerol, and lactic acid. We achieved complete utilization of these four major hydrolysate substrates achieving productivities of 0.037 (g/L/h) from *Scenedesmus obliquus* and 0.029 (g/L/h) from *Monoraphidium minutum* hydrolysates. Final titer and process yield (mol of muconic acid per mol of substrate (molP/molS)) were 0.99 g/L and 0.42 molP/molS from *S. obliquus* hydrolysate and 0.75 g/L and 0.23 molP/molS from *M. minutum* hydrolysate, respectively.

Materials and Methods

- *Pseudomonas putida* KT2440 (CJ522) was engineered to produce muconic acid
- The culture was revived in a 125 mL baffled shake flask with 25 mL of LB. Inoculum cultures were grown aerobically at 30° C at 225 rpm for 18 hours and used to inoculate the fermenter at a starting OD 600 nm of 0.1.
- M9 media was used for culturing, inoculum preparation, and as a media experimental control to determine baseline muconic acid production. The M9-base media consisted of 13.56 g/L Na_2HPO_4, 6 g/L KH_2PO_4, 1 g/L NaCl, 2.25 g/L $(NH_4)_2SO_4$, 0.24 g/L $MgSO_4$, 0.011 g/L $CaCl_2$, and 0.0027 g/L $FeSO_4$. This media was supplemented with 5 g/L yeast extract and 10 /L peptone (0.5x YP), 1 g/L glucose (G) and 1 g/L mannose (M) (M9YPGM).
- Pretreated algae liquor was fermented using 125 mL baffled shake flasks (25 mL of working volume). The flasks were wrapped in aluminum foil to prevent light from catalyzing the epimerization of cis, cis to cis, trans muconic acid. Shake flasks were incubated using the same conditions as those to generate the inoculum. Each shake flask received 20 mL of PAL, 5 mL of M9-base media and 1.1 mL of inoculum.
- Two fast-growing algae species, *Scenedesmus obliquus* and *Monoraphidium minutum*, were cultivated at the Arizona Center for Algae Technology and Innovation Cultivation and harvesting of algae cultures was performed in raceway ponds. Pretreatment of the algae biomass was performed in a 4 L (1 L working volume) batch-type ZipperClave® reactor. For this study, 300 g of wet algal paste was loaded into the reaction chamber along with H_2SO_4 and water to achieve a final solids loading of 18% (w/w) and an acid concentration of 2.0% (w/w) of the liquid. The hydrolysates were neutralized to pH 7.0 using sodium hydroxide prior to fermentation.
- The lactic acid media consisted of M9-based media and 3 g/L of lactic acid as the only carbon source. The fermentation was conducted the same as previously described.

Conclusion

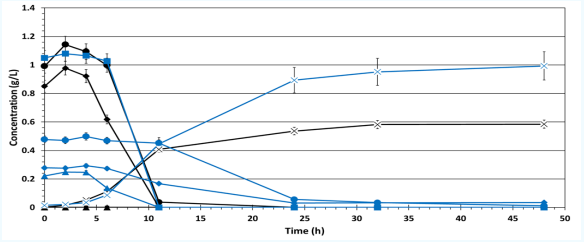
Muconic acid was successfully produced from, high-protein algal biomass thus expanding the suite of potential feedstocks amenable to fermentation in CAP valorization of algal biomass. *S. obliquus* and *M. minutum*, were pretreated to produce PAL for fermentation. During fermentation, sugar utilization was rapid and complete for the four major carbon sources in PAL demonstrating the efficacy of pretreatment and fermentation for high-protein algae biomass feedstocks. While the concept of implementing high protein algal biomass in the existing CAP was somewhat successful, either the low carbohydrate content in these feedstocks needs to be increased or genetic engineering to drive carbon flux from amino acids into the muconic acid pathway needs to be realized for economic feasibility.

Compositional Analysis of Algae Biomass

Algae Species	FAME Lipids	Ash	Carbohydrates	Protein	Mass Balance	Glucose	Mannose	Galactose	Fucose	Total Carbohydrates
S. obliquus	7.96	7.31	10.23	48.52	74.03	3.05	4.95	1.57	0.49	10.23
M. minutum	9.45	6.70	13.54	41.54	71.23	4.89	6.32	1.75	0.09	13.54

Reported as percent of total dry mass, percent mass balance closure, and percent of total carbohydrates

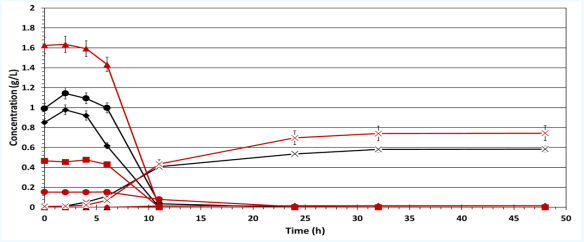
Fermentation profile for *S. obliquus* PAL



S. obliquus PAL (blue line), control media (black line),
glucose (♦), lactic acid (▲), glycerol (■), mannose (●), and muconic acid (✕)

- The overall productivity was 0.037 (g muconic acid / L h) with a yield of 0.42 (mol muconic acid / mol initial substrate)
- Fermentation proceeded very rapidly demonstrating the non-toxic and highly fermentable nature of pretreated algal biomass
- These results indicate that algae hydrolysate is a suitable substrate for *P. putida* and that muconic acid can be integrated as a product into a biorefinery based on CAP

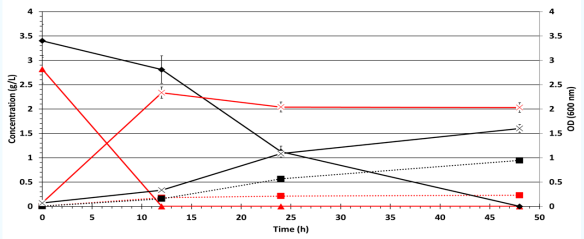
Fermentation profile for *M. minutum* PAL



M. minutum PAL (red line), control media (black line),
glucose (♦), lactic acid (▲), glycerol (■), mannose (●), and muconic acid (✕).

- The overall productivity was 0.029 (g muconic acid / L h) with a yield of 0.23 (mol muconic acid / mol initial substrate)
- While the initial glucose and mannose concentrations are higher in the control, the muconic acid titer produced was the lowest
- The productivity and yield of the lab media control were lower than either of the algae liquors

Muconic Acid Production from Lactic Acid as the Only Substrate



Lactic acid media (red line), control media (black line),
glucose (♦), muconic acid (■ and dotted line), lactic acid (▲), optical density (OD600) (✕).

- The overall productivity was 0.0034 (g muconic acid / L h) with a yield of 0.055 (mol muconic acid / mol initial substrate)
- The media that contained lactic acid produced a higher OD much more quickly than the media that contained glucose as the carbon source
- While process yields from lactic acid as a substrate are lower, utilization was extremely rapid compared to glucose

This work was authored by the National Renewable Energy Laboratory, operated by Alliance for Sustainable Energy, LLC, for the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding provided by U.S. Department of Energy Office of Energy Efficiency and Renewable Energy Bioenergy Technologies Office . The views expressed in the article do not necessarily represent the views of the DOE or the U.S. Government. The U.S. Government retains and the publisher, by accepting the article for publication, acknowledges that the U.S. Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this work, or allow others to do so, for U.S. Government purposes.

44th Symposium on Biotechnology for Fuels & Chemicals
New Orleans, LA
May 1 - May 4, 2022
NREL/PO-2700-82803