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# *Production of a biofuel intermediate from biomass sugars by Clostridium tyrobutyricum*

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## Abstract

Butyric acid is a valuable chemical that can be fermentatively produced in *Clostridium tyrobutyricum* and catalytically upgraded to drop in biofuels. Despite considerable study of glucose fermentation in *C. tyrobutyricum*, information about glucose and xylose co-fermentation to produce butyric acid is limited. In this work, fed-batch fermentations of corn stover hydrolysate at pH 5.0 demonstrated the co-fermentation of glucose and xylose. However, the fermentation stalled at approximately 48 hours after reaching inhibitory levels of butyric acid (18 g/L). When the fermentation pH was increased to 6.0, higher butyric acid titers (50 g/L) were achieved but the fermentation again stalled after 98 hours due to product inhibition. To demonstrate the potential of a continuous co-fermentation process, in situ product recovery via membrane-based liquid-liquid extraction (pertraction) was applied to remove butyric acid. The extracted acids were then recovered from the organic solvent via back extraction into an alkaline solution, producing a high purity product. Results from pertractive fermentations of real corn stover hydrolysate are shown below.

## Materials/Methods

### Fermentation and seed media :

*C. tyrobutyricum* (ATCC25755) fermentations were conducted in the presence of various sugar sources in 'Clostridium Media' (CM) media which contained (g/L): yeast extract, 10; peptone, 5; $KH_2PO_4$, 3.26; cysteine-HCl, 0.5; $CaCl_2{\cdot}2H_2O$, 0.02; $MgSO_4{\cdot}7H_2O$, 0.3; $MnSO_4{\cdot}7H_2O$, 0.02; $FeSO_4{\cdot}7H_2O$, 0.03; $(NH_4)_2SO_4$, 3.0 resazurin, 0.001. The media utilized to revive the bacterium from glycerol stocks was Reinforced Clostridial Medium (Becton Dickenson, Franklin Lakes, NJ) modified to contain 10 g/L glucose and 20 g/L xylose (mRCM). The seed media utilized to inoculate the bioreactors was CM media with 10 g/L glucose and 40 g/L xylose.

mRCM was autoclaved in the absence of sugars. When the media cooled down, sugars were added. All other media was sterile filtered inside an anaerobic chamber. Serum bottles containing mRCM were filled and sealed in the anaerobic chamber.

DDR hydrolysate sugars were produced from high solids enzymatic hydrolysis using corn stover substrate that is deacetylated with dilute sodium hydroxide at 92 C followed by disk refining using 36" Andritz double disk refiner at a refining energy of 200 kWh/ tonne of biomass. DDR sugars were added to CM media at the appropriate concentrations.

### Seed culture preparation:

Revive was prepared by adding 1 vial of frozen stock to a serum bottle containing 50 mL of mRCM and incubated at 37 ºC and 100 rpm for 18 h. This culture was used to inoculate a 0.5 L seed fermentor at an initial $OD_{600}$ of 0.1. The seed was controlled at 37 ºC, pH 6.0 (NaOH) and 150 rpm for 24 h to a final $OD_{600}$ of ~20.

### Fed-batch fermentations:

Fed-batch fermentation started with CM and 50 g/L of sugars (glucose, xylose, and arabinose) obtained from enzyme hydrolysis of deacetylated and disk refined corn stover (DDR). Feed contained DDR at about 500 g/L fermentable sugars in addition to CM media components (x1). Continuous feed began when glucose concentration was near 0 g/L. Feed rate was adjusted to maintain glucose <10g/L.

### Fed-batch, pertractive fermentations:

The pertractive fermentation system consisted of two Liqui-Cel Extra-Flow 2.5x8 membrane contactor units (3M, Charlotte, NC), a 3-L BioFlow 3000 fermentor, three peristaltic pumps, flow meters, pressure gauges, and valves. Batch media consisted of 1.6 L of CM media with DDR hydrolysate at 50 g/L fermentable sugars. The fermentation conditions were: initial pH was 6.0, and controlled at 5.0 with ammonium hydroxide, 37 C, and 150 rpm agitation. The fermentation broth was continuously circulated through the lumen side of the first membrane contactor at 40 mL/min. The organic extractant was circulated through the shell or lumen side at 20 mL/min. The 2.0 N NaOH stripping solution was circulated through the shell side of the second membrane contactor at 40 mL/min. Needle valves were used to maintain 3–4 psi higher pressure on the aqueous side of the membrane. The system was sterilized with 100% isopropanol, drained, and dried overnight with sterile air.

### Analysis of sugars and carboxylic acids:

Sugars were measured by an ICS-5000+ system consisting of an AS-AP autosampler, and a pulsed electrochemical detector with a gold electrode and an Ag/AgCl reference electrode (Dionex Corp., Sunnyvale, CA, USA). Samples were diluted to a quantifiable range and 10 µL was injected on to a CarboPac SA-10 Dionex carbohydrates column (4 x 250 mm) paired with a CarboPac SA-10 guard column (4 x 50mm). Separation was with an isocratic flow of 1 mM potassium hydroxide at 1.5 mL $min^{-1}$ for 15 min at 45° C

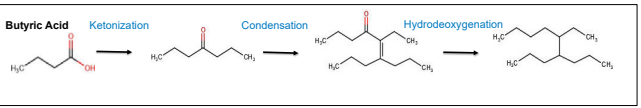
Carboxylic acids were measured by HPLC using an Aminex HPX-87H (300x7.8mm) organic acid column and Cation H+ guard. The column temp was 65C and mobile phase consisted of 0.01 N sulfuric acid and a flow rate of 0.6 mL/min.

## References

1. Huo X., et. al. *Fuel Property First: Tailoring Diesel Bioblendstock from Integrated Catalytic Upgrading of Carboxylic Acids. Under review.*
2. Saboe, P. O., et al. (2018). *In situ recovery of bio-based carboxylic acids.Green Chemistry 20(8): 1791-1804.*
3. *2017 BETO Peer Review*, Catalytic Upgrading of Biochemical Intermediates, https://www.energy.gov/sites/prod/files/2017/05/f34/thermochem_elander_2.3.1.100-103.pdf

## Materials/Methods

- **Butyric acid is the primary product of *C. tyrobutyricum***
- **Butyric acid can be catalytically upgraded to a diesel like biofuel [1]**




## Fed-Batch Fermentations

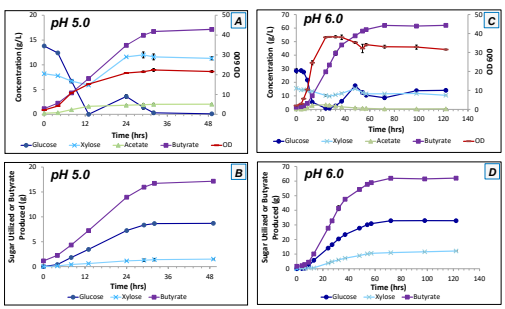



**Figure 1: Fed-Batch fermentations of *C. tyrobutyricum* in DDR: (A) Fermentation profile (A) and (B) sugar utilization at pH 5.0. (C) Fermentation profile and (D) sugar utilization at pH 6.0**

At pH 5.0, co-fermentation of glucose and xylose is seen but the fermentation ends due to butyric acid accumulation. Butyric acid is more toxic at lower pH. At pH 6.0, co-fermentation is more robust. However, fermentation still ends due to product toxicity.

Recovery of the protonated acid is required for catalytic upgrading. Despite high titers in fed-batch fermentations, the cost of solid-liquid separation, dewatering and salt breaking is too expensive. *In situ* product removal by pertraction is a solution for both the recovery of butyric acid in the acid form as well as decreasing product toxicity in the bioreactor.

## Fed-Batch Pertractive Fermentation

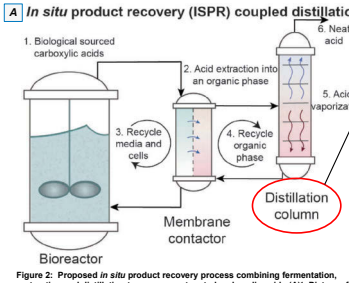



**Figure 2: Proposed *in situ* product recovery process combining fermentation, pertraction and distillation to recover protonated carboxylic acids (A)[1]. Picture of the current pertractive system using NaOH back stripping in place of distillation (B).**

For this work, integrated distillation column is not yet operational and is replaced with NaOH back extraction


- Acids produced in fermentation are extracted into the organic phase.
- Use of base for pH adjustment is minimized.
- Only protonated acids are extracted.
- The acids can then be distilled from organic phase, recovering pure, protonated acids[2].

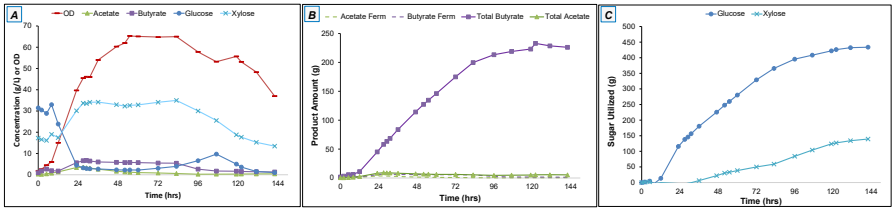



**Figure 3: Fed-Batch pertractive fermentation of DDR Hydrolysate at pH 5.0: (A) Fermentation profile in the bioreactor, (B) Carboxylic acids production, showing both acids remaining in the fermentor and extracted acids. (C) Sugar utilization profile showing glucose and xylose co-fermentation.**

| Parameter | Units | Value |
|---|---|---|
| Butyrate Produced | g | 246 |
| Butyrate Product Concentration | g/L | 54 |
| Butyrate Volumetric Production | g/L | 84 |
| Butyrate Productivity at 119h | g/L/hr | 0.70 |
| Butyrate Yield at 119h | g/g | 0.45 |

**Table 1: Mass of butyric acid produced, mass butyric acid produced per final fermentor volume, productivity and yield from a fed-batch pertractive fermentation of DDR hydrolysate at pH 5.0. Productivity and yield are calculate at 119 hours using a fermentor volume of 2.96L. Butyrate volumetric production is the total butyrate produced divided by final fermentor volume (2.96L). Butyrate product concentration is the final concentration of the recovered, extracted product.**

## Future Research

- Improve xylose utilization and other attributes through strain engineering.
- Understand and prevent cell death observed starting at about 80 hours.
- Integrate fermentation, pertraction and distillation.

## Conclusions

- **Fed-batch, pertractive fermentations with *C. tyrobutyricum* produces very high quantities of butyric acid from a single fermentation.**
- **This is the first study that reports glucose and xylose co-fermentation of biomass hydrolysate by wild type *C. tyrobutyricum*, in a non-immobilized, free cell, pertractive system at pH 5.0.**

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