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# Comparative analysis of microbial community of novel lactic acid fermentation inoculated with different undefined mixed cultures

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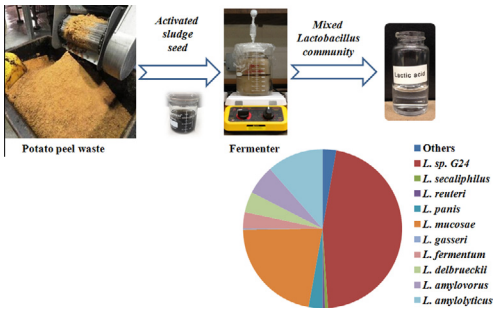
## HIGHLIGHTS

- Lactic acid was produced predominately from potato peel wastes by fermentation.
- Anaerobic sequencing batch bioreactors were seeded using mixed microbial cultures.
- Microbial community variation was monitored using 16s rRNA Illumina sequencing.
- *Lactobacillus* was the dominant group in all bioreactors and varied at species level.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Three undefined mixed cultures (activated sludge) from different municipal wastewater treatment plants were used as seeds in a novel lactic acid fermentation process fed with potato peel waste (PPW). Anaerobic sequencing batch fermenters were run under identical conditions to produce predominantly lactic acid. Illumina sequencing was used to examine the 16S rRNA genes of bacteria in the three seeds and fermenters. Results showed that the structure of microbial communities of three seeds were different. All three fermentation products had unique community structures that were dominated (>96%) by species of the genus *Lactobacillus*, while members of this genus constituted <0.1% in seeds. The species of *Lactobacillus* sp. differed among the three fermentations. Results of this study suggest the structure of microbial communities in lactic acid fermentation of PPW with undefined mixed cultures were robust and resilient, which provided engineering prospects for the microbial utilization of carbohydrate wastes to produce lactic acid.



## 1. Introduction

Lactic acid is an important industrial chemical with various applications in food, pharmaceutical, textile, leather, and other chemical industries, including for the production of a biodegradable plastic, polylactic acid (PLA) (John et al., 2007). Currently, nearly all the lactic acid manufactured is based on carbohydrate fermentation (Datta and Henry, 2006), where pure strains of lactic acid producing microorganisms such as *Lactobacillus* sp. are usually employed. Lactic acid bacteria (LAB) are aerotolerant, Gram-positive, non-spore forming, non-respiring cocci or rods, and acid tolerant bacteria that can ferment hexose sugars to produce lactic acid

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(Axelsson, 2004). Lactic acid production using LAB species can be classified into two pathways; homofermentative LAB catabolize glucose exclusively into lactic acid via the Embden–Meyerhof–Parnas pathway, while the heterofermentative LAB metabolize glucose to form lactic acid, $CO_2$, acetic acid and ethanol (Kandler, 1983; Wee et al., 2006).

A relative high yield and optically pure lactic acid can be biosynthesized through pure culture fermentation, where the sterile conditions, bacterial cell density, pH, nutrients, and temperature are critical to fermentation productivity (Zhang et al., 2007). The mixed cultures fermentation can minimize these restrictions with the highest microbial diversity and adaption abilities and the production of value added carboxylic acids and methane have been widely studied using undefined mixed cultures (activated sludge) from municipal wastewater treatment plants (Agler et al., 2012; Coats et al., 2013; Forrest et al., 2012; Werner et al., 2011). Activated sludge is a highly complex biological mixture with bacteria as the dominant group, but also contains eukaryotes, archaea, and viruses as determined by conventional molecular techniques and next-generation sequencing methods (Dai et al., 2015; Ju et al., 2013; Wang et al., 2012a; Zhang et al., 2012). These mixed microbial cultures can be used as seeds in biological conversion processes for the production of specific organic compounds such as volatile fatty acids (VFAs) and lactic acid by adjustment of feeding and operational parameters (Bengtsson et al., 2008; Liang et al., 2014). Previously work has demonstrated the production of lactic acid from potato peel waste (PPW) with undefined mixed cultures by batch fermentation (Liang et al., 2014).

In this study, activated sludge samples were collected from three municipal wastewater treatment plants located in different geographic regions of the United States and used as fermentation inocula to examine the robustness and resilience of microbial communities in this novel lactic acid fermentation process. The chemical properties of the three fermentation broths and the microbial community structures of seed cultures and fermented biomass samples were characterized using Illumina sequencing technology. The objective of this study was to address the following two questions. (i) Do different seed cultures affect the production of lactic acid from PPW? and (ii) will the diversity of the microbial community be reduced and become more homogeneous during fermentation?

## 2. Methods

### 2.1. Feedstock, mixed cultures and fermentation

The PPW feedstock used in this experiment was obtained from a potato processing plant of JR Simplot Company (Caldwell, ID) and stored at −20 °C in Ziploc bags. Detailed chemical compositional analysis was conducted according to the analytical methods described previously (Liang and McDonald, 2014), and results showed that the PPW contained 16.8% starch, 7.8% cellulose, 14.7% hemicellulose, 25.4% protein, 2.0% lipids, 21.6% lignin and suberin complex, and 11.1% ash (dry basis).

The seed cultures used in fermentation experiments were obtained from the aeration tank (Moscow, ID) and return activated sludge tank (Boise, ID and West Lafayette, IN) of municipal wastewater treatment plants. Table 1 describes the basic characteristics of the three municipal wastewater treatment plants. The three sludge samples were collected on 6/11/2013, 6/12/2013, and 6/18/2013, respectively, and kept chilled in plastic bottles and couriered overnight to laboratory. Once received the samples were divided into two portions: one portion was used immediately for the fermentation experiments and the other portion was kept at −20 °C for genomic DNA extraction and analysis.

The fermentation experiments were conducted in three independent glass vessels with working volumes of 800 mL, maintained at 35 °C, and sealed with a fermentation air-lock. The bioreactors were inoculated with 5% (v/v) seeds from the municipal wastewater treatment plants and labeled as Reactor B0, M0, and W0 (Boise, Moscow, and West Lafayette, respectively). The reactors were fed daily with the same amount of PPW that had first been gelatinized/sterilized by placed it in a pressure cooker at 121 °C for 30 min to kill bacteria in PPW, and then cooled down. The solid feed loading was 30 g/L and the solid/hydraulic retention time (SRT/HRT) was 2 d. The reactors were stirred and cycled daily and operated for at least 12 d (6 SRT/HRT cycles) to ensure steady state. After that the fermentation broth and biomass samples for each bioreactor (labeled B1, M1, and W1) were collected in three successive days and the mixed biomass samples were stored at −20 °C.

### 2.2. Characterization of fermentation broth

The fermentation broth samples were centrifuged at 12,000 rpm for 5 min and the clear supernatants were analyzed according to the methods described below. The pH was measured with an Orion-3-Star DO/pH portable meter (Thermo Fisher Scientific Inc., Waltham, MA). Ammonium nitrogen ($NH_4$-N), total phosphorous (TP), and chemical oxygen demand (COD) were determined respectively, according to standard methods (APHA, 1998) using the Nessler method 4500-$NH_3$, ascorbic acid method 4500-P, and 5220-D with Hach high-range COD kits using a Beckman D640 spectrophotometer (Beckman Instruments, Fullerton, CA).

Lactic acid, succinic acid, and glucose were quantified by high-performance liquid chromatography (HPLC) using a Rezex ROA organic acid column ($\Phi$ 7.8 mm × 30 cm, Phenomenex, Torrance, CA) at 65 °C with a Waters HPLC pump using 0.005 N $H_2SO_4$ (0.5 mL/min) as a solvent, and a differential refractive index detector (ERC-5710, ERMA, Japan). Alcohols and VFAs in the broth were quantified by gas chromatography (GC) using an Agilent 6890 instrument (Agilent Inc., Palo Alto, CA, USA) with an Alltech-Heliflex-AT™ Wax capillary column ($\Phi$ 0.32 mm × 30 m, Grace Davison Discovery Sciences, Deerfield, IL, USA) at 150 °C and a flame ionization detector. The injector and detector temperatures were maintained at 210 °C with He as a carrier gas, and all the samples were acidified to pH 2 with HCl prior to injection.

The D- and L-lactic acid ratio was determined by the method (Inoue et al., 2007) described as: clear supernatant samples (1 mL) were acidified to pH 2 with HCl and extracted with ethyl acetate (4 mL). The extracted mixed solution and (−)-menthol solution (100 μL, 200 mg/mL in ethyl acetate) were evaporated to remove the ethyl acetate under a stream of $N_2$ at 37 °C for

**Table 1**
Characteristics of three municipal wastewater treatment plants used as inoculum sources.

| Code | Location | Process configuration | Average influent flow rate ($m^3$/d) | Sampling point |
|---|---|---|---|---|
| B | Boise, ID | Nitrifying activated sludge | 53,800 | Return activated sludge tank |
| M | Moscow, ID | Hybrid A2/O process with oxidation ditch | 11,400 | Aeration tank |
| W | West Lafayette, IN | Nitrifying activated sludge | 24,600 | Return activated sludge tank |