



# Fungal biofermentation of pine bark producing organic acids and their quantification with capillary electrophoresis



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## ABSTRACT

Bioprocessing of pine bark was accomplished using oyster mushroom fungi. The organic acids and inorganic anions in the extracts of fermented samples were identified by capillary electrophoresis (CE). Nine organic acids were simultaneously determined in 12 min through the use of an electrolyte composed of 20 mM pyrazine-2,3-dicarboxylic acid (2,3-PyDC), 1.5 mM CaCl<sub>2</sub>·2H<sub>2</sub>O and 0.3 mM MTAH in methanol:water (10:90, v/v) at pH 9.0. The concurrent determinations of three inorganic anions were performed in less than 10 min in an electrolyte consisting of 2.25 mM pyromellitic acid, 6.50 mM NaOH, 0.75 mM hexamethonium hydroxide, and 1.60 mM triethanolamine at pH 7.70. The applied analysis methods efficiently separated and identified organic acids and inorganic anions in the extracts. Butyric, maleic and glucuronic acids were the primary organic acids formed via fermentation. Chloride, nitrate and sulphate were detected in all of the samples.

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## 1. Introduction

A biorefinery is a facility that carries out the bio-based production of power, fuels, and chemicals in an integrated manner (NREL, 2009). The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum (NREL, 2009). In general, biorefinery involves the conversion of carbohydrates, lignins, oils and proteins that exist in raw biomaterials to useful intermediates or final products via biochemical and chemical methods (Kamm and Kamm, 2007). Currently, research, development and industrial implementation efforts are primarily focused on green biorefineries (green crops and green biomass), the two-platform concept (sugar and synthesis gas platforms), whole-crop biorefineries (cereals) and lignocellulosic biorefineries (wood, forest residues, paper-waste and straw) (Kamm and Kamm, 2007).

A majority of the current biorefineries produce biofuels and chemicals from sugarcane and corn (C6 sugars), which are food and feed at the same time (Koivistoinen, 2013). Therefore, there has been growing interest in using non-food carbon sources, such as lignocellulosic materials, being feedstock in these biorefineries. Forestry residues, which are lignocellulose-based raw materials,




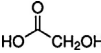
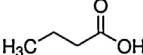

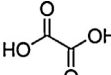
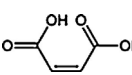
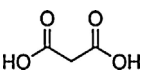

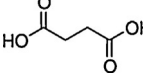
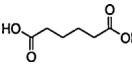
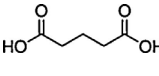
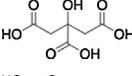
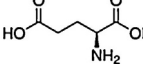

constitute one of the largest sources of renewable biomass still accessible to humans (Barton, 1985).

Lignocellulose is the largest component (app. 90%) of plant matter, making up the cell walls of woody plants, such as trees, shrubs and grasses (Brandt et al., 2013). Lignocellulose is composed of lignin (aromatic polymer), cellulose and hemicellulose (chain polymers) (Arkenol, 1996). Fungi, bacteria or yeast can disintegrate lignocellulose. Fungi have two types of enzymatic systems: a hydrolytic system producing hydrolases that degrade polysaccharide and an oxidative and ligninolytic system that degrades lignin (Sánchez et al., 2009). White- or brown-rot fungi, filamentous fungi and oyster mushroom fungi (*Pleurotus ostreatus*) were isolated and used in the degradation of lignocellulosic biomass (Sánchez et al., 2009; Galkin et al., 1998). In particular, Finnish pine bark was inoculated using white-rot fungi (Valentín et al., 2010). After 90 days of inoculation, the original content of Klason lignin, cellulose and hemicellulose (i.e., app. 45, 25 and 15%, respectively) was reduced to app. 39, 5 and 5%, respectively, indicating that cellulose was the most degradable natural polymer using that type of fungi (Valentín et al., 2010). Both cellulose and hemicellulose are long chain polymers consisting of individual sugar molecules (Arkenol, 1996). During bioprocessing, enzymatic attack depolymerises chains back to their constituent sugars. Therefore, the cellulose chain breaks down to glucose (C6), and hemicellulose disintegrates to xylose (C5) (Arkenol, 1996). The degradation of xylose can produce alcohols, organic acids, polyols, ketones and gases (Turkia et al., 2010).

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**Table 1**  
Organic acids and their chemical structures.

Organic acid	Molecular weight (g/mol)	Structure	Organic acid	Molecular weight (g/mol)	Structure
Formic acid	46.03		Acetic acid	60.05	
Propionic acid	74.08		Glycolic acid	76.05	
Butyric acid	88.11		L-Lactic acid	90.08	
Oxalic acid	90.03		Maleic acid	116.07	
Malonic acid	104.06		Benzoic	122.12	
Succinic acid	118.09		Adipinic	146.14	
Glutaric	132.12		Citric acid	192.12	
L-Glutamic	147.13		D-Glucuronic acid	194.140	

Microbial production of organic acids from renewable carbon sources may be a potentially viable alternative to obtain building block chemicals (Sauer et al., 2008). Building block chemicals are molecules containing multiple functional groups that can be converted to molecular precursors for the new materials (Sauer et al., 2008). In 2008, small industrial-scale plants produced lactic acid and citric acid (150,000 and 1,600,000 t, respectively) using glucose as a carbon source and lactic acid bacteria and filamentous fungus *Aspergillus Niger* as microbial sources, respectively (Sauer et al., 2008). There are also promising research studies that have demonstrated the production of organic acids using lignocellulosic feedstock and bacteria (Rovio et al., 2010; Turkia et al., 2010).

Fungi have the ability to produce a variety of organic acids (Karaffa et al., 2001). Fungi have an inherent capability to accumulate the compounds, which is most likely due to this accumulation affording them an ecological advantage as they grow fairly well at pH 3–5 and survive even at pH 1.5 (Karaffa et al., 2001). The production of organic acids from non-food carbon sources using fungi has been rarely reported (Tsao, 1963; Galkin et al., 1998). The wood-rotting fungus *P. ostreatus* NRRL 2366 produced oxalic acid from mixtures of sawdust and CaCO<sub>3</sub> as well as from the solid residue of the acid hydrolysis of wood when inorganic nutrients were added into the culture (Tsao, 1963). Cultivation of white rot fungi in straw medium resulted in the production of oxalic acid (Galkin et al., 1998).

Bark is a forest residue obtained from the production of lumber. Previous studies employed wood bark to produce several chemicals. Pine bark was used as a source of tannins for wood adhesives (Fradinho et al., 2002). Chestnut shell and eucalyptus bark extracts were investigated as phenol substitutes in the formulation of adhesives, as chrome substitutes in leather tanning and as a source of antioxidants compounds (Vázquez et al., 2009). Condensed tannins, which are natural preservatives of ligninocellulosic materials in bark, were investigated as a source of novel biocides (Laks, 1989). Natural tannins extracted from pine bark was used in steel anti-corrosive protection (Matamal et al., 2000). We have designed the current study to produce organic acids using the pine bark and

oyster mushroom fungi considering the fact that fungi may work well in the bioconversion of lignocellulosic residues (Sánchez et al., 2009). This study is the first to produce organic acids by combining this type of carbon source and fungus.

## 2. Materials and methods

### 2.1. Materials

Fresh pine disks (diameter: 50 cm and thickness: 10 cm) were supplied by a Finnish forestry company. Bark was shaved from the disk, cut to 1 cm-pieces, grounded and kept in a cold storage to keep microbes alive. Oyster mushroom (*P. ostreatus*) fungi (Remasan capsules) was bought from a pharmacy in Lappeenranta, Finland.

### 2.2. Chemicals

All the chemicals used during analyzes were analytical grade. Table 1 shows the details of the organic acids used in the experiments. Acetic acid (glacial), oxalic acid, adipic, succinic acid, L-glutamic acid, methanol, ammonia and NaOH were purchased from Merck. D-Glucuronic acid, maleic acid, benzoic acid, glutaric acid, Na<sub>2</sub>SO<sub>4</sub>, NaCl, NaNO<sub>3</sub> and pyrazine-2,3-dicarboxylic acid (2,3-PyDC) and hexamethonium hydroxide (0.1 M in water) were sourced from Sigma-Aldrich. Malonic acid, glycolic acid, citric acid, and butyric acid were from Fluka. Formic acid, propionic acid, and triethanolamine were obtained from Baker. Lactic acid was a variable mixture of D- and L-lactic acids (BDH, AnalaR). Myristyltrimethylammonium hydroxide (MTAH) and CaCl<sub>2</sub>·H<sub>2</sub>O were purchased from Waters-Ion select, and Riedel de Haen, respectively.

### 2.3. Biosynthesis

The synthesis conditions were optimized based on preliminary experiments. Accordingly, the 130 g amount of pine bark powder

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