

BIOCONVERSION OF *CITROFORTUNELLA MICROCARPA* FRUIT WASTE INTO LACTIC ACID BY *LACTOBACILLUS PLANTARUM*

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ABSTRACT

The processing of *Citrofortunella microcarpa* fruit juice generates large volume of solid waste, causing disposal problem. Several studies have demonstrated that wastes from agricultural and food processing industries such as fruit and vegetable peels contain high amount of polysaccharides that can be transformed into useful chemicals, including lactic acid, through fermentation. Lactic acid is widely used in various industries, such as in the manufacture of biodegradable plastic, and the demand for this chemical justifies the search of renewable feedstock for its biotechnological production. This study aimed to produce lactic acid from *C. microcarpa* fruit waste biomass through fermentation with *Lactobacillus plantarum*. The hydrolysate from *C. microcarpa* fruit waste was prepared, inoculated with different amounts of *L. plantarum* cell suspension, and incubated for three days. Lactic acid production was monitored daily. The lactic acid produced from the fermentation was recovered as calcium lactate and lactic acid crystals. The identity of the crystals was evaluated using Fourier transform infrared spectroscopy (FTIR) spectroscopy and paper chromatography. The highest lactic acid production was observed in fermentation mixtures containing the highest number of *L. plantarum* cells. Within three days of fermentation, the amount of lactic acid production increased with increasing period of incubation. Partial characterization of the crystals recovered from the fermentation mixtures by FTIR spectroscopy showed that the peaks in the spectrum were consistent with the chemical structure of lactate. Paper chromatography results likewise confirmed that the crystals are lactate. *C. microcarpa* fruit waste can afford lactic acid when fermented with *L. plantarum*. The results of the study may serve as basis for the development of technology for the utilization of *C. microcarpa* fruit waste biomass as renewable resource for industrial production of lactic acid.

Keywords: *Lactobacillus plantarum*, *Citrofortunella microcarpa*, lactic acid; waste valorization; bioconversion

INTRODUCTION

Citrofortunella microcarpa is among the most widely cultivated fruit crop in the Philippines and disposal of wastes from its processing may pose a serious environmental problem. The juice from the *C. microcarpa* fruit is extracted and used as a common flavoring agent in Philippine cuisine. It is also processed industrially into bottled or

packed juice drink products, and incorporated in various consumer products including flavored soy sauce, detergents and dishwashing liquid. Data from the Philippine Statistic Authority show that in the last quarter of 2016, the country's production of *C. microcarpa* fruits reached an estimated 28.13 tons (<https://psa.gov.ph/fruits-crops-bulletin/calamansi>). Thus, it can be assumed that tons of wastes consisting of fruit peels and seeds

are also produced from the industrial processing of *C. microcarpa* fruit juice.

Waste valorization, or the conversion of wastes into useful products (Arancon, Lin, Chan, Kwan, & Luque, 2013), is an alternative management strategy to deal with the disposal challenges posed by wastes from food and agricultural industries. Because these wastes contain large amount of sugars that can be easily assimilated by microorganisms, they can be transformed into industrially important chemicals (Couto, 2008). For example, citric acid has been produced through the fermentation of banana (Karthikeyan & Sivakumar, 2010) and orange peel (Rivas, Torrado, Torre, Converti, & Dominguez, 2008), both through the action of *Aspergillus niger* while butanol has been obtained from the fermentation of apple and pear peels by *Clostridium acetobutylicum* (Raganati, Procentese, Oliveri, Russo, & Marzocchella, 2016).

Among the industrial chemical that may be obtained from fruit and vegetable wastes is lactic acid. Lactic acid has numerous applications in the food and cosmetics industries as well as in the manufacture of biodegradable plastics (Datta & Henry, 2006) and as a precursor of other chemicals and biocompatible materials with medical applications (Martinez, et al., 2013). A common route for the production of lactic acid is through the hydrolysis of lactonitrile, which is obtained from the reaction between acetaldehyde and hydrogen cyanide (Huang, Jin, Lant, & Zhou, 2003; Martinez, et al., 2013). Lactic acid may also be produced from fermentation of various materials consisting of monosaccharides and disaccharides, starchy materials, and lignocellulosic hydrolyzates by microorganisms such as lactic acid bacteria (Martinez, et al., 2013). Previous studies have shown that lactic acid can be produced from mango peel waste by a consortium of indigenous microorganisms (Jawad, Alkarkhi, Jason, Easa, & Nirulaini, 2013) and from potato wastewater with the aid of *Rhizopus arrhizus* (Huang, Jin, Lant, & Zhou, 2003). Various microorganisms have also been shown to be effective in converting different raw materials into lactic acid. These include organisms of the following genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Clostridium* and *Weissella* (Huang, Jin, Lant, & Zhou, 2003). Lactic acid has been produced by the following species: *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus delbrukei* subsp

bulgaricus, and *Bifidobacterium bifidum* (Chick, Shin, & Ustunol, 2001) and *Lactobacillus plantarum* (Sreenath, Moldes, Koegel, & Straub, 2001).

It has previously been demonstrated that the fruit peel of *C. microcarpa* has volatile oil and the residue left after the extraction of oil can be used in the production of pectin (Anzaldo & Briones, 1993). The fruit peel of *C. microcarpa* contains 16.12 g total sugar per 100 g (Samonte & Trinidad, 2013) and is therefore a potential substrate for the microbial production of lactic acid. However, to the best of our knowledge the production of lactic acid from the waste from the processing of *C. microcarpa* fruit has never been attempted. Therefore, this study was conducted to investigate the potential of *C. microcarpa* fruit waste as substrate for the biotechnological production of lactic acid.

METHODOLOGY

The waste materials produced after the expression of the juice from *Citrofortunella microcarpa* fruits were collected from food vendors inside the Central Luzon State University campus in Nueva Ecija, Philippines. The seeds were removed before the rest of the waste (rind, pulp and juice vesicles) were cleaned of unwanted materials by washing with distilled water. The cleaned waste was used in the production of hydrolysate through steam explosion by modifying a previously described method (Pumiput, Chuntanuluck, Kitpreechavanich, Punsuvon, & Vaithanomsat, 2008). Forty grams of *C. microcarpa* fruit waste was steam-exploded in an autoclave at 121°C for 20 min. Enough sterilized water was added to the steam-exploded waste to a volume of 1 L, and then the mixture was heated for 20 min at 80°C. The solid materials were separated from the supernatant with a cheese cloth. Acid hydrolysis was carried out by adding HCl (RCI Labscan, Thailand) to the supernatant to a final concentration of 1% v/v and then autoclaving the mixture at 121°C for 30 min (Umesh & Preethi, 2014). The pH of the hydrolysate was adjusted to 6–6.8 using CaO (Sigma-Aldrich, USA) and the CaSO₄ precipitate was separated from the hydrolysate through filtration (Mudaliyar, Sharma, & Kulkarni, 2012).

Lactobacillus plantarum (BIOTECH 1223) was obtained from the National Institute of Molecular Biology and Biotechnology, University of the Philippines at Los Baños College, Laguna, Philippines. The bacterium was first grown on

solid de Man Rogosa (MRS) medium (Scharlau, Spain) before the preparation of inoculum according to a previously described method (Umesh & Preethi, 2014) where *L. plantarum* was cultivated in MRS broth prepared with the hydrolysate. After incubation for three days in a microaerophilic environment, the cell density was adjusted to 1×10^7 cfu/mL based on McFarland standard.

Fermentation mixtures were prepared by adding the *L. plantarum* cell suspension to the hydrolysate to produce mixtures with final concentrations of 3, 5 and 10%. A negative control (0% cell suspension) was also prepared. The hydrolysate-cell suspension mixtures were incubated for three days.

The daily lactic acid production in each of the mixture was estimated by titrating an aliquot against standardized 0.1N NaOH (Tehnopharmachem, India) using phenolphthalein as indicator and by applying the factor 1 mL of 1N NaOH is equivalent to 90.08 mg of lactic acid (Sheeladevi & Ramanathan, 2011).

The lactic acid in the fermentation broth (70 mL) was recovered as calcium lactate and lactic acid crystals (Evangelista, 1994). The pH of the broth was adjusted to 10–11 using calcium hydroxide (RCI Labscan, Thailand) before the temperature was raised to 80°C to 100°C. The coagulated protein and cells were removed by filtration. The filtrate containing the lactic acid was added with activated carbon to remove colored components. After filtration, the filtrate was added with 5 ml sulfuric acid (Scharlau, Spain) before undergoing a second bleaching with activated carbon. Any precipitate that formed after the addition of sulfuric acid was discarded. The excess water from the filtrate was evaporated to obtain the lactate crystals.

The production of lactate was confirmed through characterization by Fourier transform infrared spectroscopy (MB3000, Clairet Scientific, Canada) and paper chromatography using a previously described method (Al-Asady, 2012). Whatmann No. 1 served as the stationary phase while the mobile phase was 10:2 (v/v) n-butanol (Scharlau, Spain): formic acid (Sigma-Aldrich, USA) containing 0.01% bromocresol green (RCI Labscan, Thailand). Pure lactic acid (Thecnopharmachem, India) served as reference. After the development of the chromatogram, the paper was exposed to ammonia vapor. The appearance of yellow spot on a blue background indicated the presence of lactate.

Statistical analysis of the data was performed using Prism 7 (GraphPad Software, La Jolla, USA).

RESULTS AND DISCUSSION

All fermentation mixtures, except the negative control, produced lactic acid (Figure 1). The results suggest that *C. microcarpa* fruit waste can serve as substrate for lactic acid production by *L. plantarum*. It was observed that the production of lactic acid increased with increasing *L. plantarum* cell density in the fermentation mixture and with longer fermentation period. The highest amount of lactic acid was obtained in the mixture containing 10% cell suspension and incubated for three days.

The statistical analysis of the data (two-way ANOVA) also revealed a significant interaction ($P < 0.0001$) between *L. plantarum* cell density and period of incubation. This implies that lactic acid production in mixtures with different cell content depends on the number of days of incubation, while the changes in lactic acid production at each day depends on the concentration of cells in the fermentation mixture.

The lactic acid produced from the fermentation of *C. microcarpa* fruit waste with *L. plantarum* was precipitated. The highest amount of crystals was obtained in the set up with 10% bacterial cell and that was incubated for three days (Figure 2). As expected, the trend in the production of lactate crystals was consistent with that observed for the bioconversion of sugars in the hydrolysate to lactic acid. Characterization of the crystals by Fourier transform infrared spectroscopy (Figure 3) revealed the presence of peaks for O-H (3379 cm^{-1}), C=O (1612 cm^{-1}) and C-O (1080.06 cm^{-1}) in the spectrum that are consistent with the functional groups expected in the structure of lactate (Coates, 2000). The qualitative test by paper chromatography also showed that the crystal was a lactate (Figure 4).

Although the results do suggest that *C. microcarpa* fruit waste can be valorized as feedstock for lactic acid production, the optimum conditions for the bioconversion process have not been investigated in the present study and future investigations must focus on process optimization. For example, studies may be designed to find the optimum initial number of bacterial cell to hydrolysate ratio that would yield the highest amount of lactic acid. Such information is need-

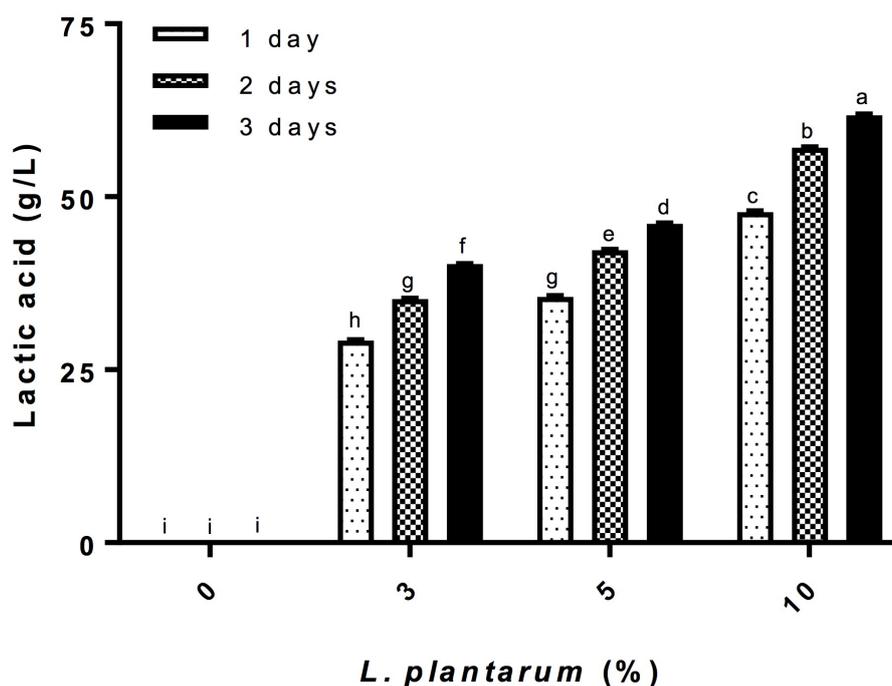


Figure 1. Lactic acid produced by *Lactobacillus plantarum* from *Citrofortunella microcarpa* fruit biomass hydrolysate. The data are presented as mean (error bar = SD) of three observations. Columns labeled with the same letter are not significantly different from each other based on a two-way ANOVA followed by Tukey’s multiple comparison test ($p < 0.0001$). The interaction between day of incubation and *L. plantarum* cell density was found to be significant ($p < 0.0001$)

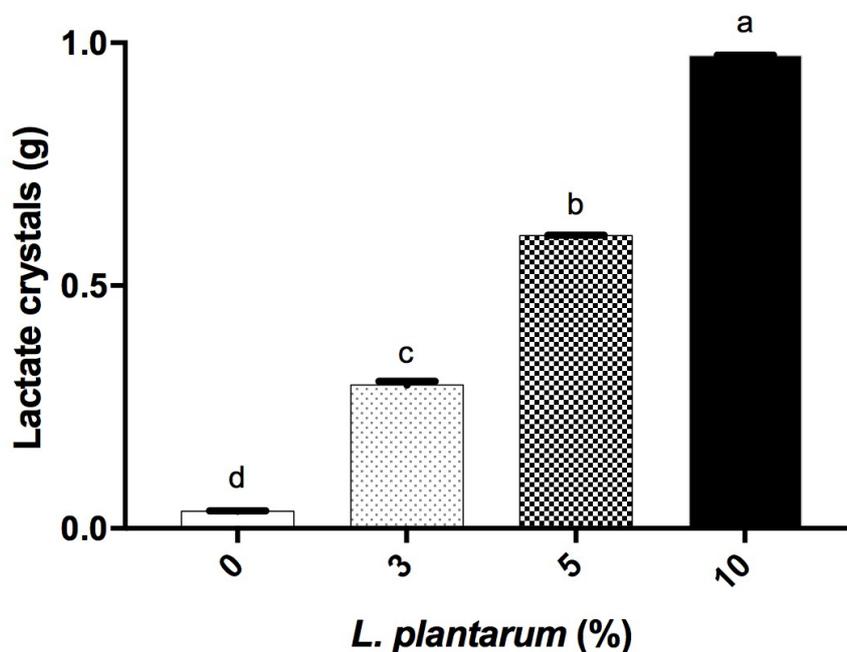


Figure 2. Lactate crystals recovered in *Citrofortunella microcarpa* hydrolysate after 3 d of fermentation with *Lactobacillus plantarum*. The data are presented as mean (error bar = SD) of three observations. Columns labeled with the same letter are not significantly different from each other based on one-way ANOVA followed by Tukey’s multiple comparison test ($p < 0.0001$)

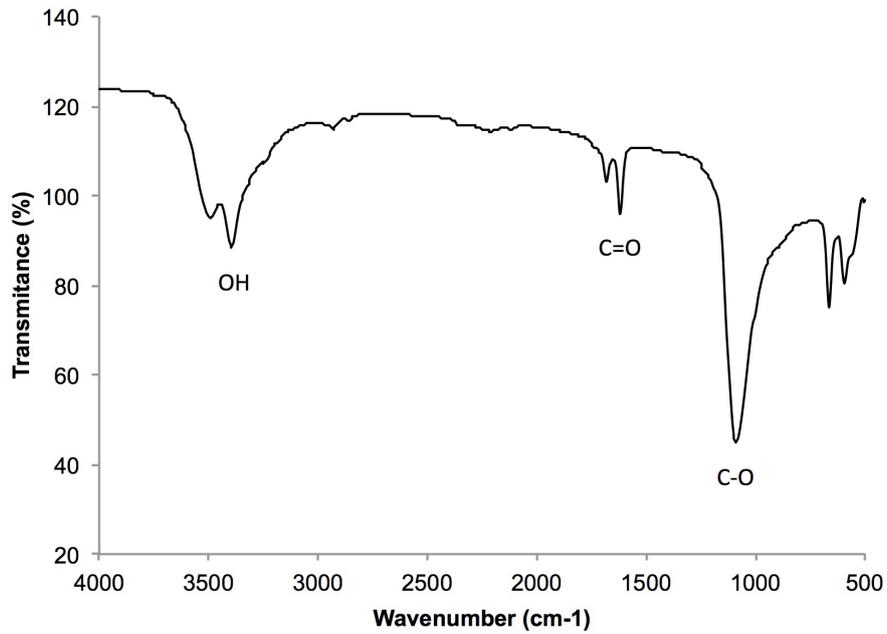


Figure 3. Infrared spectrum of lactate crystals produced by *Lactobacillus plantarum* from *Citrofortunella microcarpa* fruit waste hydrolysate

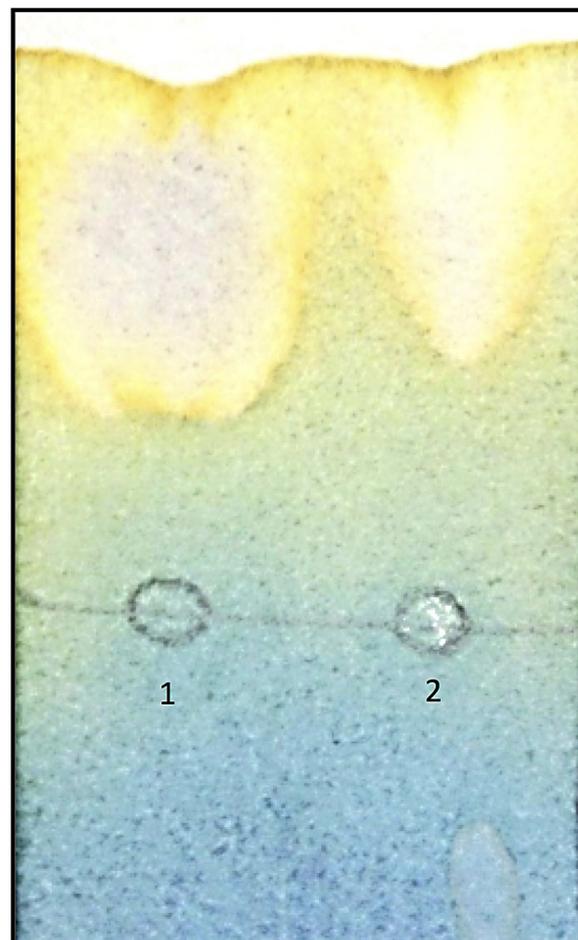


Figure 4. Representative paper chromatogram of lactic acid (1) and lactate produced by *Lactobacillus plantarum* from *Citrofortunella microcarpa* fruit waste biomass (2). The presence of yellow spot against blue background confirms the presence of lactate

ed to ensure that the cells will not be saturated with the substrate, and that lactic acid will continuously be produced as the cell population increases. Likewise, the maximum period of incubation before the production of lactic acid ceases must be determined.

Nonetheless, the findings are consistent with previous reports about the utility of fruit and vegetable wastes as substrate for the biotechnological production of lactic acid (Al-Asady, 2012; Huang, Jin, Lant, & Zhou, 2003; Jawad, Alkarkhi, Jason, Easa, & Nirulaini, 2013; Sreenath, Moldes, Koenig, & Straub, 2001) and other important chemicals, including ethanol (Karthikeyan & Sivakumar, 2010; Raganati, Procentese, Oliveri, Russo, & Marzocchella, 2016; Rivas, Torrado, Torre, Converti, & Dominguez, 2008). Large amount of waste from processing of agricultural products that is generated worldwide is underutilized and discarded, despite the presence of polysaccharides in these materials that may be transformed into saleable chemicals (Van Dyk, Gama, Morrison, Swart, & Pletschke, 2013). Use of these wastes as raw materials to produce new products is highly desirable, since it promotes the so-called zero waste economy and industrial ecology (Mirabella, Castellani, & Sala, 2014).

Due to the large demand for biodegradable polylactic acid based materials, which are alternatives to petroleum-derived plastics, there has been an increase in interest in the production of lactic acid from cheap and renewable sources (Abdel-Rahman, Tashiro, & Sonomoto, 2013). In 2009, the Philippines was the sole exporter of fresh fruits and juice concentrate of *C. microcarpa* in the world (Real & Digal, 2010). Tons of *C. microcarpa* fruits are produced annually in the country and processing of these fruits will inevitably lead to the generation of tons of wastes. Using *C. microcarpa* wastes as feedstock for lactic acid production, which this study shows to be possible, is a green option for the management of these wastes.

CONCLUSION

Large amount of waste from the processing of *C. microcarpa* fruits are produced annually, creating disposal problems. This study primarily aimed to investigate the possibility of using the waste from the processing of *C. microcarpa* fruit waste as substrate in the production of lactic acid by *L. plantarum*. Lactic acid was produced

from the hydrolysate of *C. microcarpa* fruit waste and the production of the chemical increases with increasing *L. plantarum* cell density in the fermentation broth and with increasing period of incubation. Lactic acid was recovered as crystals and characterization by FTIR spectroscopy and paper chromatography confirmed that the product was lactate. Although optimization studies have yet to be done, the results of the present study nevertheless support the mounting evidence that wastes from agriculture and food processing can be utilized for the biotechnological production of industrially important chemicals.

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