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Lactic Acid Production from Almond Hulls

Glenn G^{1*}, Thomas S¹, Franqui-Villanueva D¹, Hart-Cooper W¹ and Waggoner M²

¹USDA-ARS, 800 Buchanan Street, Albany CA 94710, USA ²Grow Plastics, USDA-ARS, 800 Buchanan Street, Albany CA 94710, USA

Abstract

Research Article

Lactic acid (LA) is a commodity chemical used in pharmaceuticals, bio plastics, and food, home and personal care products. It is commercially produced by fermentation of corn starch, which requires large amounts of land and water. Almond hulls are a cheap agricultural by product that have high sugar content and could be used as a carbon source in the fermentation of lactic acid. In this study, we fermented almond hulls using a mixed culture from primary sludge and a mono-culture from Lactobacillus rhamnosus and compared the production of lactic acid from almond hulls against that of other alternative feedstocks. Other feedstocks tested included corn stover and pine wood as lignocellulosic feedstocks, food waste, glucose, glycerol as a cheap chemical byproduct and sorbitol as a negative control. In both mixed culture and pure culture, almond hulls (maximum values for yield 0.55 g/g biomass, productivity 2.8 g/L/h, >99% L-LA) gave higher yields than food waste (maximum values for yield 0.95 g/g biomass, productivity=1.2 g/L/h, 84-95% L-LA), but lower yields than glucose (maximum values for yield 0.95 g/g biomass for yield 0.95 g/g biomass of L-LA). Pine wood and corn stover did not produce lactic acid efficiently under the mixed culture conditions tested. The results of this study lend support for the use of almond hulls as an affordable feedstock for the production of lactic acid.

Keywords: Fermentation; *Lactobacillus rhamnosus*; Poly (lactic acid); Feedstock

Introduction

Lactic acid (2-hydroxypropanoic acid) is a naturally occurring hydroxycarboxylic acid that was isolated first in 1780 from sour milk [1]. Lactic acid (LA) is a commodity chemical used in pharmaceuticals, bio plastics, and food, home and personal care products [2]. The homopolymer of lactic acid, poly (lactic acid) (PLA), is a biodegradable, transparent and inexpensive alternative to petroleum based plastics that are persistent in the environment. When used as a replacement for conventional plastic and properly composted, PLA may reduce landfill waste, plastic/micro plastic pollution and greenhouse gas emissions [3]. These characteristics have increased global demand for lactic acid from 130,000-150,000 metric tons in 1999 to 1.1 million metric tons in 2016 and demand is expected to grow further by 16.2% between 2017 and 2025 [4]. While industrial scale chemical synthesis results in racemic mixtures of lactic acid, fermentation processes may produce optically enriched lactic acid. Polymer and food applications require the highly enantioenriched isomer of lactic acid (typically >90% L-lactic acid), making fermentation from corn starch-derived sugar the preferred method of production.

Developing carbohydrate-rich feedstocks from non-food sources abates concerns about diverting food feedstocks to non-food products [5]. Producing lactic acid from waste streams such as food waste, industrial byproducts (e.g., glycerol), or lignocellulosic biomass (e.g., corn stover, pine wood and other agricultural biomass) rather than corn starch has the potential to lower production costs. It has been reported that food waste can be converted selectively to lactic acid in excellent yields [6-8]. Another abundant and inexpensive waste feedstock for lactic acid is a byproduct of almond production. Almonds are an important crop in California where more than 2.4 billion pounds of shelled nuts are produced annually comprising more than 80% of the global production [9]. Both the shell and outer leathery hull of the almond are byproducts of kernel production. The hulls comprise nearly 53% of the fresh weight of the almond fruit or approximately 9 billion pounds [10]. Almond hulls are used primarily as cattle feed for nearby dairies. However, price volatility for the hulls is a concern, such as when prices dropped 50% between 2012 and 2015 due to low milk prices [11]. As a result, value-added markets are being sought [11]. Almond hulls contain 37.3% fermentable sugars by weight, [12] making them an attractive source of cheap carbohydrates for fermentation. Production of bioethanol and biomethane from almond hulls have been demonstrated, [12] which raises the possibility that they could be utilized for lactic acid fermentation. The objective of this study was to assess almond hulls as a feedstock for lactic acid production by comparing them with alternative feedstocks including food waste, chemical feedstocks, and lignocellulosic waste.

Materials and Methods

Nonpareil almond (*Prunus dulcis* (Mill) Webb cv. Nonpareil) hulls were obtained from a commercial almond processing facility located in Chowchilla, CA. *Lactobacillus rhamnosus* (ATCC* 10863^m) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cellulase from *Trichoderma reesei* and cellobiase from *Aspergillus niger* (Novozyme 188) were purchased from Sigma-Aldrich (St. Louis, MO) for pretreating lignocellulosic waste. Other reagent grade chemicals were purchased from Sigma-Aldrich.

Feedstock preparation

Almond hull: The almond hulls were ground in a Wiley mill (Model 4, Thomas Scientific, and Ramsey, MN) to pass through a 5-mm screen. Ground almond hulls were prepared in two ways; as a lignocellulosic feedstock (see below) or as steep liquor. For almond hull steep liquor, ground almond hulls (330 g) were suspended in 2 L of tap water and mixed with an overhead laboratory mixer. The almond

*Corresponding author: Glenn G, USDA-ARS, 800 Buchanan Street, Albany CA 94710, USA, Tel: 1(510) 559-5677; E-mail: Greg.Glenn@ARS.USDA.GOV

Received December 21, 2018; Accepted January 11, 2019; Published January 11, 2019

Citation: Glenn G, Thomas S, Franqui-Villanueva D, Hart-Cooper W, Waggoner M (2019) Lactic Acid Production from Almond Hulls. J Food Ind Microbiol 5: 128. doi:10.4172/2572-4134.1000128

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hull suspension was heated to 80°C and mixed for 75 minutes then allowed to cool while covered. After cooling to room temperature, the suspension was filtered through a 1.4 mm screen and the filtrate was centrifuged at 4,000 rpm for 5 minutes. The supernatant was collected, and the solid pellet discarded. The supernatant was stored (5°C) until needed for fermentation experiments.

Food waste: Food Waste No.1 was collected randomly from a household trash bin. The waste mainly consisted of lasagna, ground beef, chicken salad, and rice. Food waste No.1 was blended prior to fermentation. Food waste No.2 was a defined waste that was formulated with the intent to emulate the composition of American food waste (Table 1) [13]. The food components were homogenized in an industrial Waring blender until a thick paste formed. The paste was blended with water (1:1).

Lignocellulosics: The method for the saccharification of the lignocellulosic feedstocks was adapted from an established procedure [14]. The lignocellulosic feedstocks (corn stover, pine wood and almond hulls) were ground to pass through a 5 mm screen as previously described. The ground material (30 g) was pretreated for 20 hours in 300 mL of 1 N NaOH. Each treatment had its pH adjusted to 5.5 using 50% (w/w) H_2SO_4 , before being autoclaved at 121°C for 20 minutes. After cooling, 1 mL of Cellulase from *Trichoderma reesei* and 1 mL Novozyme 188 were added to each sample. The samples were kept at 50°C in a shaking incubator (120 rpm) for 3 days before being used for fermentation.

Ingredient	Mass (g)
Vegetable oil	262.5
Bread	266.4
Yellow corn tortillas	220.1
Pink lady apples	486.5
Whole carrots	332.5
Russet potato	332.5
Tongol tuna	94.0
Cooked chicken breast	308.5
Unsalted peanuts	14.1
Chicken eggs	73.5
Whole milk	668.5
Sucrose	441.0

 Table 1: Food waste no. 2 formulation.

Sludge inoculated fermentation

The method for the sludge-inoculated fermentation was adapted from a previous study [7] using a citric acid buffer to control the pH. Each fermentation broth consisted of 5.0 g yeast extract, 0.025 g $MnSO_4$, 0.379 g Citric Acid, 0.116 g Sodium Citrate and 220 mL tap water in a 250 mL glass media bottle. The liquor (220 mL) was used in place of the tap water for almond hull steep liquor fermentation experiments. Each treatment then received substrate to achieve the various concentrations listed in Table 2.

Primary Sludge was obtained from East Bay Municipal Utilities District wastewater treatment facility in Oakland, CA. Thirty mL of the primary sludge was added to each prepared substrate sample and sample pH was adjusted to 6.0 with 1 N NaOH. The samples were placed in an orbital shaker for 192 hours at 40°C with continuous shaking (180 rpm). Samples were taken at two intervals (0 and 192 hours) and stored (-20°C) until analyzed.

pH-controlled fermentation

pH-controlled fermentation feedstock preparation: Glucose, glycerol, and sorbitol feedstocks were prepared in concentrations listed in Table 3. A sample of each feedstock (350 mL) was supplemented with yeast extract (8.0 g) and $MnSO_4$ (0.04 g) prior to autoclaving at 121°C for 20 minutes.

Seed culture: A purchased culture of *Lactobacillus rhamnosus* was supplied as a pellet that was rehydrated with MRS broth and incubated at 37°C. Overnight cultures were prepared by inoculating 50 mL of MRS broth with a glycerol stock of *L. rhamnosus* and placed in an incubator (37°C) overnight. In the morning, the cultures were centrifuged at 5,000 rpm for 5 min. After discarding the supernatant, the pellet was washed and resuspended in 50 mL deionized water.

Fermentation protocol: Each prepared broth was fermented in a 500 mL glass media bottle placed inside a water bath on a digital hot plate/stirrer. The water bath was maintained at 37°C while a stir bar placed inside of the broth rotated at 200 rpm. The pH of the solution was maintained between 5.5 and 6.0 with 1 N NaOH that was added by a peristaltic pump controlled by an automatic titrator (Milwaukee Instruments MC122 pH controller). The bottle was sealed by a cap and septa, through which the pH probe and NaOH supply line were placed.

Feedstocks	Initial feedstock (g/L)	LA Concen. (g.L)	Yield (g/g)	Enantio-selectivity (% L-LA)	Initial pH	Final pH
Glucose	44.2	29.6	0.67	47.9	6.08	3.43
Glycerol	44.0	9.7	0.22	28.0	6.05	3.88
Sorbitol	43.5	12.5	0.29	60.9	6.01	3.78
Almond hulls	116	11.6	0.10	50.4	6.03	4.10
Almond hull steep liquor	95.5	18.3	0.19	52.4	6.12	4.18
Food waste no.1	74.8	22.5	0.30	47.0	6.09	3.72
Corn stover	58.6	13.9	0.24	62.0	6.08	4.13
Pine wood	76.8	0.0	0.00	N/A	6.05	6.55
Hydrolyzed almond hulls	75.1	20.2	0.27	57.6	6.14	4.03

Table 2: Lactic acid (LA) fermentation of waste feedstock's using sludge inoculum and no pH control.

Feedstock	Initial feedstock (g/L)	Sugars (%)	Yield LA (g/g)	Time (h)	Productivity (g/L/h)	Enantio-selectivity (%L-LA)
Glucose	46.8	100	0.95	26.0	1.71	96.0
Glucose no.2	45.9	100	0.70	10.0	3.21	97.6
Glucose – high conc.	100	100	0.73	17.5	4.17	96.7

Conditions: 37°C, 5.5<pH<6.0, stirred 120 rpm

Table 3: pH controlled lactic acid fermentation of glucose using Lactobacillus rhamnosus.

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The fermentation roths were inoculated with 50 mL of the washed and resuspended cultures of *Lactobacillus rhamnosus*. The 500 mL glass media bottle containing the broth was connected to the pH controller and placed in the water bath. The amount of NaOH added to the broth was monitored by weight changes in the NaOH bottle using data logging software (Balance Link by Mettler Toledo). Samples of the feedstock were removed at intervals and frozen at -20 °C for later analysis by high pressure liquid chromatography (HPLC). The batches were terminated when the pH controller finished adding NaOH.

HPLC

Samples (1 mL) were centrifuged at 5,000 rpm for 5 min. The supernatant was filtered into vials using a 0.2 μ m polyvinylidene fluoride (PVDF) filter (Thomson Instrument Company, Clear Brook, VA). Lactic Acid concentration was measured by HPLC using an Agilent 1200 system with an Aminex HPx 87H column and a refractive index detector (flow rate: 0.5 mL/min at 40°C with a 0.01 N H₂SO₄ mobile phase). Glucose, fructose, xylose, ethanol, and sucrose concentrations were determined by HPLC using an Aminex HPx 87P column and a refractive index detector (flow rate: 0.5 mL/min at 85°C, with water as mobile phase).

Optical purity

Optical purity was measured using the Megazyme D-Lactic Acid and L-Lactic Assay Kit [15]. The samples were centrifuged at 14,000 rpm for 5 minutes after which the supernatant was analyzed according the manual assay procedure for the sequential assay of D-lactic acid and L-lactic acid included with the kit.

Results and Discussion

Sludge inoculated fermentation

Waste materials such as almond hulls provide a vast resource for value added products through fermentation. Others have investigated energy-rich waste feedstocks that are typically landfilled, such as food waste that releases methane into the environment [16]. Food waste can be efficiently fermented into lactic acid with good yields [7]. The results of the present study confirm that various waste feedstocks can produce lactic acid using municipal sludge as the inoculum (Table 2). Glucose proved to be the most efficient carbon source for lactic acid production from sludge inoculum with yields of 67%. Feedstocks such as pine wood contain 45-50% of cellulose by dry weight [17]. Although cellulose is a polymer of glucose, the cellulase pretreatment of the pine wood was not effective in digesting the cellulose into glucose as evident by the lack of lactic acid produced from this feedstock. It may be that longer pretreatment periods or a wider array of cell wall degrading enzymes are needed to breakdown the pine wood into fermentable sugars.

The yield of lactic acid was comparable for glycerol, sorbitol, and food waste or corn stover as feedstocks (Table 2). Solid, unprocessed almond hulls had the lowest lactic acid yield of the almond hull-derived samples tested. The LA yield was nearly doubled by using the almond hull steep liquor as a feedstock. The highest lactic acid yield from almond hulls was achieved from hydrolyzed almond hulls, which were pretreated with NaOH followed by cellulose. The cost of pretreating almond hulls for sugar extraction must be factored in when evaluating which of the three almond hull feedstocks is most economically viable.

Lactic acid enantioselectivity from the mixed inoculum was poor, between 28 and 62% L-lactic acid (Table 2). The low enantioselectivity was likely caused by the wide diversity of microbes in the sludge that provided little control over which enantiomers were produced. The glycerol feedstock notably produced the highest enantioselectivity from sludge treatment (72% D-lactic acid), suggesting that the microbes capable of glycerol metabolism selectively produced this enantiomer.

The fermentations described in Table 2 afforded lower yields than those previously reported [7]. It was hypothesized that these low yields were due to the acidification of the fermentation liquid (final pH to 3.43 for the glucose feedstock and 3.72 for food waste). Previous studies have shown that a drop in pH can arrest microbial activity resulting in low yields [18]. The combination of low yield, low pH, and poor enantioselectivity warranted pH control and a more enantioselective inoculum.

pH-controlled monoculture fermentation

To improve these low yields and poor enantioselectivities (Table 2), different feedstocks were fermented under pH control and with pure culture. The pH was kept above 5.5, by a pH controller to prevent inhibition of *L. rhamnosus* growth. With pH kept above 5.5, the lactic acid yield increased for the glucose feedstock (Table 3) and over 95% of the lactic acid produced was the L-enantiomer. A second trial of glucose was run but with a shorter fermentation time (10 h). The shorter fermentation time nearly doubled the productivity of the fermentation process although yields were lower (Table 3). Increasing the glucose concentration in the fermentation liquid only slightly improved the yield of lactic acid but productivity was further increased.

The pH-controlled fermentation of almond hull steep liquor using *L. rhamnosus* inoculum resulted in a much higher yield of L-lactic acid compared to the sludge inoculum compare Tables 2 and 4. Furthermore, the yield was nearly three times higher under pH control. As with the glucose feedstock, productivity using the almond hull steep liquor improved by reducing the time of fermentation. The lactic acid productivity was nearly three times higher by reducing the fermentation time by almost four-fold. Yields were also decreased by reducing the increase in productivity (Table 4).

Food waste No.1 had much better yields and productivity when supplemented with yeast extract and $MnSO_4$ Table 5. Without the nutrient supplementation, food waste No.1 was a very poor feedstock for lactic acid. Food waste No.2 was a defined waste used without nutrient supplementation. Interestingly, the percentage of L-lactic acid as well as the yield and productivity were lower in food waste No.2 compared to food waste No.1 with nutrient supplementation. Increasing the amount of food waste No.2 and the reaction time increased the yields and surprisingly, also increased the productivity, albeit not to the level of food waste No.1 with supplementation.

Feedstock	Initial feedstock (g/L)	Sugars (%)	Yield LA (g/g)	Time (h)	Productivity (g/L/h)	Enantio-selectivity (%L-LA)
Almond hull SL*	82.6	63.1	0.55	48.0	0.95	100
Almond hull SL no.2	97.0	41.4	0.47	23.0	1.98	96.1
Almond hull SL no.3	96.0	n.d.	0.37	12.8	2.78	97.6

*SL=Steep Liquor; Conditions: 37°C, 5.5<pH<6.0, stirred 120 rpm; n.d.: not determined

Table 4: pH controlled lactic acid fermentation of almond hull using Lactobacillus rhamnosus.

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Feedstock	Initial feedstock (g/L)	Sugars (%)	Yield LA (g/g)	Time (h)	Productivity (g/L/h)	Enantio-Selectivity (%L-LA)
Food waste no.1+S*	74.8	33.1	0.44	28.0	1.18	95.0
Food waste no.1-S	74.8	5.9	0.10	28.0	0.27	n.d.
Food waste no.2 trial no.1	129.6	43.0	0.12	29.0	0.54	79.9
Food waste no.2 trial no.2	139.6	40.6	0.22	45.2	0.68	76.1
Food waste no.2 trial no.3	164.9	34.1	0.16	37.8	0.70	84.3

*S=yeast extract + MnSO₄; Conditions: 37°C, 5.5<pH<6.0, stirred 120 rpm; n.d.: not determined

Table 5: pH controlled lactic acid fermentation of food waste using Lactobacillus rhamnosus.

Feedstock	Initial feedstock (g/L)	Sugars (%)	Yield LA (g/g)	Time (h)	Productivity (g/L/h)	Enantio-Selectivity (%L-LA)
Almond hull SL*	91.9	52.3	0.46	27.9	1.52	97.9
Food waste no.2	145	39.2	0.17	37.3	0.66	80.1
Sorbitol	43.5	0.0	0.18	27.0	0.29	96.9
Glycerol	45.1	0.0	0.01	20.0	0.023	n.d.

*SL=Steep Liquor. Conditions: 40°C, 5.5<pH<6.0, stirred 180 rpm; n.d.: not determined

Table 6: pH controlled lactic acid fermentation of feedstocks using Lactobacillus rhamnosus.

A set of fermentation experiments were conducted using almond hull steep liquor, food waste No.2, and the two chemical feedstocks sorbitol and glycerol, Table 6 tested with sludge inoculum (Table 2). Almond hull steep liquor served as the best feedstock relative to the other feedstocks tested using the inoculum of *L. rhamnosus* and controlling the pH (Table 6). The percentage of L-lactic acid was high, and the yield and productivity were higher with the almond hull steep liquor. Many bacteria lack the ability to efficiently metabolize either glycerol or sorbitol, which appeared to be true for *L. rhamnosus* [15]. The glycerol feedstock produced virtually no lactic acid over a 20 h fermentation trial. Sorbitol was comparable to food waste No.2 with regards to yield but productivity was lower. However, sorbitol produced a very high percentage of L-lactic acid compared to the food waste.

The results of the present study show that enantioselectivity of the feedstocks improved with the use of pure culture, as *L. Rhamnosus* almost exclusively produces lactic acid as the L-enantiomer. All the feedstocks except Food waste No.2 saw marked improvement towards L-lactic acid production, with higher than 95% L-Lactic acid being produced. These improvements suggested that controlling pH and using pure culture are superior for producing L-lactic acid.

Under the pH control and pure culture conditions, the almond hull steep liquor feedstock resulted in higher yields, concentrations, and productivities compared to the glycerol, sorbitol, and food waste feedstocks, although still lower in comparison to the glucose control. While the almond hull steep liquor performed well under these conditions, it was formed under minimal water conditions and would need to be concentrated to achieve higher concentrations in the fermentation.

A simulated food waste (food waste No.2) was fermented to examine its performance under pH control and inoculation by pure culture. Lower lactic acid concentrations, yields, and productivities were observed in comparison to the almond hull steep liquor feedstock. The food waste batches required longer retention times than the almond hull steep liquor feedstocks due to their low productivities. The poor performance of the food waste feedstock could be due to carbon catabolite repression [19] due to the complex composition of the food waste substrate, which is known to reduce yield and productivity of fermentation. There was great variability between the 2 food waste feedstocks that we tested; suggesting that the food waste composition greatly influences the ability to produce lactic acid [20]. For both food waste and almond hulls, it is uncertain if the higher costs of purifying the lactic acid and effluent treatment would allow for the profitable production of lactic acid. Future studies could focus on optimizing the conditions tested here to improve productivity and yield, as well as reducing variability among batches. Purification and separation of lactic acid from the fermentation liquid would also be required to help understand the viability of commercially producing lactic acid from waste feedstocks.

Conclusion

Almond hulls can be utilized as an alternative substrate to corn starch in the production of lactic acid. The use of the non-food agricultural by product instead of corn starch could allow farmland waste to be used more productively and could reduce water usage that is inherent in commercial production of poly (lactic acid). Depending on the price of almond hulls, it could also be a cheap source of carbohydrates for other fermentation systems. Glycerol and sorbitol performed poorly with pure culture but could be produced with sludge in higher amounts if enantioselectivity is not an issue.

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