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Research Article

Lactic Acid Yield Using Different Bacterial Strains, Its Purification, and Polymerization through Ring-Opening Reactions

F. G. Orozco,[1] A. Valadez-González,[1] J. A. Domínguez-Maldonado,[2] F. Zuluaga,[3] L. E. Figueroa-Oyosa,[2] and L. M. Alzate-Gaviria[2]

[1] *Materiales Unidades, Centro de Investigación Científica de Yucatán (CICY), Calle 40 No. 130, Colonia Chuburná de Hidalgo, 97200 Mérida, YUC, Mexico*
[2] *Renovables Unidad de Energía (CICY), Calle 40 No. 130, Colonia Chuburná de Hidalgo, 97200 Mérida, YUC, Mexico*
[3] *Laboratorio de Polymer Group SIMERQO, Universidad del Valle, Ciudad Universitaria, Meléndez, 25360 Cali, Valle del Cauca, Colombia*

Correspondence should be addressed to L. M. Alzate-Gaviria; lag@cicy.mx

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Laboratory-scale anaerobic fermentation was performed to obtain lactic acid from lactose, using five lactic acid bacteria: *Lactococcus lactis, Lactobacillus bulgaricus, L. delbrueckii, L. plantarum,* and *L. delbrueckii lactis*. A yield of 0.99 g lactic acid/g lactose was obtained with *L. delbrueckii*, from which a final concentration of 80.95 g/L aqueous solution was obtained through microfiltration, nanofiltration, and inverse osmosis membranes. The lactic acid was polymerized by means of ring-opening reactions (ROP) to obtain poly-DL-lactic acid (PDLLA), with a viscosity average molecular weight (Mv) of 19,264 g/mol.

1. Introduction

The quantity of nonbiodegradable plastic waste and the recycling of this waste pose increasing problems due to the loss of properties during each processing cycle, meaning that it is important to make products with biodegradable materials both in order to maintain basic needs and for waste processing.

A fully biodegradable polymer is a material that is converted to carbon dioxide, water, minerals, and biomass by organisms with no environmental impact or ecotoxicity [1]. Polylactic acid (PLA) is a biodegradable, biocompatible, and compostable polyester that can be used to manufacture biomedical scaffold implants and bone cements, surgical materials, and commercial disposable materials such as cups, plates, cutlery, and food containers. PLA possesses similar mechanical properties and processing conditions to polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS) [2].

The monomer employed for PLA synthesis is lactic acid, which is a byproduct of anaerobic fermentation of an organic substrate transformed by microorganisms of the *Lactobacillus* genus [3]. Soccol et al. [4] reported that the most important isomer in the food industry is *L*(+), given that it is the only one assimilated by humans through the production of the *L*-lactate dehydrogenase enzyme.

The yields obtained in different studies using lactic acid bacteria [5–12] are shown in Table 1.

Lactic acid exists in two enantiomeric forms, the *D*(+) configuration and the naturally occurring *L*(−) configuration. They produce the corresponding enantiomeric polymers by conservation of the chiral center. Commercial PLAs are also copolymers of *L*-lactide and *D*-lactide and their optical purity strongly affects their properties. Optically pure PLA is isotactic and highly crystalline. Decreasing the optical purity reduces the degree of stereoregularity and crystallinity. Poly(*L*-lactide) with more than 15 mol% *D*-lactide is mostly amorphous [13].

Table 1: Yields obtained in different fermentation conditions using lactic acid bacteria.

LAB	System	pH/T°C	Yield*	Reference
L. plantarum	Batch	6.0/35	0.84	[5]
L. helveticus	Continuous	5.5/42	0.84	[6]
L. delbrueckii lactis	Batch	6.2/37	0.96	[7]
L. delbrueckii	Batch	5/50	0.92	[8]
L. delbrueckii lactis	Batch	5.5/42	0.9	[9]
Lactococcus Lactis	Batch	6.0/38	0.94	[10]
L. bulgaricus	Batch	5.5–6.0/38	0.98	[11]
L. plantarum	Batch	6.4/42	1.5	[12]

LAB: lactic acid bacteria; T: temperature; *Yield (grams/gram) of lactic acid from lactose.

Monomer purity is important for polymerization to occur with good yields in terms of molecular weight and the desired characteristics for processing and application. A number of techniques have been employed in this vein, such as solvent extraction [14], ionic adsorption, direct distillation and membrane technology. The latter employs filtration systems and has proven fruitful in the fields of separation and concentration. It has numerous advantages over several traditional separation techniques, such as solvent extraction, adsorption and direct distillation. Furthermore, it offers greater energy efficiency and the high cost of solvents and adsorbents is not required in membrane separation, which allows for the possibility of concentrating organic compounds [15]. The technique for separating lactic acid by nanofiltration is a high-pressure solution diffusion mechanism and is suitable for molecules within the range of 50–500 Da. It produces monomers with purity levels of over 80% [16].

Lactic acid polymerization can be performed by means of opening the ring of the lactide, composed of two lactic acid molecules that form a ring in the presence of tin salts, heat, and vacuum [17]. ROP of lactones has been widely studied over the past 40 years. Carothers and coworkers explored this technique for lactones, anhydrides, and carbonates [18]. In the present day, the process for obtaining PLA via ROP employs polycondensation to obtain low molecular weight PLA, depolymerization to form the cyclic dimer (lactide), and ring-opening polymerization to obtain high molecular weight polylactic acid with tin(II) octoate or another organic stannous salt as a catalyst. As far as the polycondensation reaction is concerned, the prepolymer formed must have a molecular weight between 500 and 1000 Da, given that molecular weights below 500 do not favor the formation of the cyclic dimer, and molecular weights greater than 1000 lead to transport phenomena problems due to the increased viscosity [19].

The aim of this work was to compare the lactic acid yield from powdered milk using different lactic acid bacteria, its purification, through nanofiltration and inverse osmosis, and the synthesis of PDLLA using ring-opening polymerization.

2. Material and Methods

2.1. Substrate and Microorganisms. Lactose was obtained from powdered milk at concentration of 50 g/L.

The strains of *L. delbrueckii lactis*, *L. plantarum,* and *L. delbrueckii* were acquired from the National Collection of Microbial Strains and Cell Cultures of CINVESTAV, Mexico City, Mexico, and the strains of *L. bulgaricus* and *Lactococcus lactis* from Distribuidora Alcatraz, S.A. de C.V., Danisco brand.

2.2. Culture Medium. The culture medium used was rich in lactose, 15 g milk powder per 100 mL water, and MRS (Man, Rogosa, and Sharpe) agar. The growth curve was produced using threaded tubes with 10 mL of lactose broth for each strain in triplicate based on the McFarland scale at 600 nm.

2.3. Fermentation. The fermentation broth was prepared from 15 g milk powder resuspended in 100 mL distilled water (equivalent to 50 g/L lactose), enriched with yeast extract and 1% bacteriological peptone. The pH was adjusted with 4 N NaOH to 6.0 for *Lc. lactis*, *L. delbrueckii*, and *L. delbrueckii lactis*, 6.5 for *L. plantarum,* and 5.5 for *L. bulgaricus*. The fermentation conditions were 38°C and 150 rpm under N_2 atmosphere.

2.4. Separation and Concentration. The fermentation broth was centrifuged at 400 rpm and subsequently filtered in a 100 to 150 mL/min cross flow cell. Microfiltration employed Whatman 0.22 μm cellulose filter paper. Nanofiltration (NF) was performed with a NF-5 Sepro membrane under a 100 torr vacuum and reverse osmosis with a RO-4 membrane and 10 torr vacuum.

2.5. Ring-Opening Polymerization. PLA synthesis was carried out with the lactic acid obtained from the *L. delbrueckii* fermentation process using ROP polymerization. A temperature of 170°C and a 120 torr vacuum were applied for 3 hours for polycondensation. Once the product was weighed, 1% by weight anhydrous tin(II) chloride was added to produce lactide via inverse sublimation, with a temperature of 220°C and a 60 torr vacuum. The lactide was recrystallized in ethyl acetate five times at 70°C and stored in a vacuum desiccator for 24 hours [20]. Polymerization of the lactide was performed using tin octoate at 130°C for 24 hours.

2.6. Analysis. The growth curves were produced using a Jenway 6405 UV-Vis spectrophotometer.

Concentration and lactose consumption during fermentation were determined with the dinitrosalicylic acid (DNS) method [21]. The *D*- and *L*-lactic acid yield and its concentration were estimated during fermentation with a Boehringer Mannheim/R-Biopharm enzymatic kit.

The polymer was characterized with Fourier Transform Infrared Spectroscopy (FTIR) using a Nicolet Protégé 460 Magna IR spectrophotometer. Molecular weight was determined by capillary viscometry using a Ubbelohde viscometer, with chloroform as solvent and a temperature of 25°C in accordance with ASTM Standard D2857 [22]. The glass

Table 2: Production of *D*- and *L*-lactic acid by bacterial strain.

($g\,L^{-1}$)	*L. bulgaricus*	*Lc. lactis*	*L. plantarum*	*L. delbrueckii*	*L. delbrueckii lactis*
D-lactic	12.35	15.40	23.87	23.44	33.42
L-lactic	15.55	14.89	22.11	27.49	7.60
Concentration	27.90	30.29	45.98	50.93	41.02
Yield*	0.52	0.55	0.94	0.99	0.93

*Yield (grams/gram) of lactic acid from lactose.

transition temperature (T_g) was estimated via differential scanning calorimetry (DSC) using a PerkinElmer DSC-7 from 20 to 170°C with an increase of 2°C/min. The Nuclear Magnetic Resonance (NMR) analysis used ^1H and ^{13}C spectra in a Bruker apparatus with $CDCl_3$ solvent at 400 MHz.

Table 3: Concentration of lactic acid by filtration processes.

($g\,L^{-1}$)	MF	NF	RO
D-Lactic acid	23.34	31.15	38.23
L-Lactic acid	27.81	34.20	42.72
Lactic acid	51.15	65.35	80.95

MF: microfiltration; NF: nanofiltration; RO: Reverse Osmosis.

3. Results and Discussion

3.1. Lag and Exponential Phase (Data Not Shown). According to the linear regression of the McFarland scale, the *L. delbrueckii lactis* and *Lc. lactis* strains have a lag phase of less than 6 hours and the exponential phase extends up to 30 hours. Measurements showed no changes in absorbance from this time up to 48 hours. The bacteria are therefore assumed to have been in the stationary phase. Bai et al. [7] obtained growth curves with exponential phases of 20 hours for *L. delbrueckii lactis* in MRS medium and Nancib et al. [10] reported an exponential phase from 2 to 12 hours for *Lc. lactis* when fermenting date juice. These variations are due to the substrate type, given that simple sugars enter the metabolic pathway directly.

In the case of *L. plantarum* and *L. bulgaricus*, the lag phase was 5 hours and the exponential phase extended to 25 hours, from which time a stationary phase was observed. Brinques et al. [12] worked with lactose broth fermented by *L. plantarum*, which presented an exponential phase from the beginning of the fermentation up to 20 hours, whilst Welman and Maddox [23] used *L. bulgaricus* to ferment a lactose-rich medium and obtained an exponential phase from 8 to 20 hours.

L. delbrueckii presented a lag phase of 6 hours, exponential phase from 6 to 18 hours, and a stationary phase from 18 to 36 hours. This behavior was similar to that obtained by Kadam et al. [24], who used sugar cane juice and noted that this strain has greater metabolic activity, which favors its growth in less time.

3.2. Fermentation. The LAB *Lc. lactis* and *L. bulgaricus* consumed the lactose in 56 and 59 h, respectively. Both were rejected due to the poor yield that they produced. 30.29 g/L lactic acid (yield: 0.55 g lactic acid/g lactose) was obtained with *Lc. lactis* and 27.90 g/L lactic acid (yield: 0.52 g lactic acid/g lactose) for *L. bulgaricus*. This quantity was similar to that produced by lactobacilli when working with cultures mixed with *S. thermophilus*, as performed by Tanaka et al. [25] with a yield of 0.68 g lactic acid/g lactose and Gueguim-Kana et al. [26] with a yield of 0.75 g lactic acid/g lactose. Both works exceeded the lactic acid concentration produced in this study, because the origin of mixed cultures can be an important factor in terms of the ability to perform lactic fermentation [27].

L. plantarum consumed the greatest quantity of lactose in 45 h, with a lactic acid concentration of 45.98 g/L (yield: 0.94 g lactic acid/g lactose). This behavior was similar to the *L. delbrueckii* strain, which produced 50.93 g/L (yield: 0.99 g lactic acid/g lactose) in 48 h. Both LAB presented similar fermentation times and substrate conversion to those reported by Brinques et al. [12], who worked with *L. plantarum*, and Kadam et al. [24] with *L. delbrueckii*, obtaining yields of 1.08 and 0.97 g lactic acid/g lactose, respectively, both in 48 h.

Meanwhile, for *L. delbrueckii lactis,* the maximum lactose consumption time was 73 h to give a total of 41.02 g/L lactic acid (yield: 0.93 g lactic acid/g lactose). Both Bai et al. [7], with a yield of 0.97 g lactic acid/g lactose, and Lee [9], with a yield of 0.70 g lactic acid/g lactose, performed fermentations in 100 h.

Bozoglu and Ray [27] and Okano et al. [28] note that some LAB families are capable of producing one or other lactic acid isomer in greater concentrations. *L. delbrueckii* and *L. delbrueckii lactis* have a greater quantity of subtypes that produce almost entirely *L*-lactic acid and *D*-lactic acid, respectively, whilst the remaining LAB used in this study produce a racemic mixture (*D,L*-lactic acid). Table 2 shows the lactic acid isomers produced by fermentation with the strains used in this study.

3.3. Separation and Concentration. Lactic acid, produced by *L. delbrueckii,* purification, and concentration was accomplished by means of microfiltration, nanofiltration, and inverse osmosis membranes. The changes in concentration for each step are shown in Table 3. The microfiltration process removed particles greater than 0.22 μm in size, clarifying the fermentation broth. When the permeate passed through the nanofiltration membrane, it remained yellow in color but appeared more translucent. Milcent and Carrere [29] worked with microfiltration using a pore size of 0.1 μm and achieved full microorganism retention, thereby clarifying the fermentation broth. The yellow coloration disappeared

almost completely when using the reverse osmosis membrane, where the lactic acid remained in the residual liquid, whilst the salts were eliminated in the permeate. The final product had an oily consistency.

The nanofiltration process concentrated the lactic acid from 51.15 to 65.35 g/L, as shown in Table 3. Li et al. [30] reported an increase in lactic acid concentration from 54 to 70 g/L based on filtration with a 200 Da membrane. This study used 250 Da, which resulted in the lower acid concentration.

Membrane yield was calculated based on permeate flow (PF) using the following equation:

$$\mathrm{PF} = \frac{\text{Permeate Volume}}{\text{Membrane Area} \times \text{Time}}, \quad (1)$$

expressed in $\mathrm{L/m^2\,h}$.

For the nanofiltration membrane, the permeate volume was 10 mL in 5 hours. The PF can be increased, without affecting lactic acid recovery and concentration, by using a design that increases the membrane area and applying pressure, as performed by Jeantet et al. [31], who worked with a spiral design at a pressure of 4.0–6.0 MPa and achieved 20 $\mathrm{L/m^2\,h}$. Li et al. [30], meanwhile, used an area of 140 $\mathrm{cm^2}$ in a cross flow module at pressures of 7.0 MPa and obtained 37 $\mathrm{L/m^2\,h}$. Lactic acid separation was not affected in either case and the recovery time was less than the one reported in this study.

A reverse osmosis membrane was employed into order to achieve a greater lactic acid concentration, resulting in 81 g/L lactic acid. Li et al. [30] achieved 100% lactic acid recovery using reverse osmosis membranes at a pressure of 5.5 MPa.

3.4. Ring-Opening Polymerization. Low molecular weight PLA was obtained from the lactic acid produced by fermentation with *L. delbrueckii* by means of direct condensation with the application of a vacuum (120 torr) and temperature (170°C). The yield obtained was 51.44%, producing a white, hygroscopic, and ductile oligomer. Lactide crystals were synthesized from the oligomer using $SnCl_2$ as catalyst at 220°C and 60 torr. Then the lactide was ring-opening polymerized to synthetize PDLLA. The efficiency of this reaction was 1.8 : 1 lactide/PDLLA.

A sample was taken in each polymerization stage to perform FTIR (Figure 1), where the –OH bond stretching can be observed at wavenumber 3469 $\mathrm{cm^{-1}}$, the stretching vibration of the –C=O bond at 1741 $\mathrm{cm^{-1}}$ and the $\mathrm{–C–O^-}$ bond at 1392 $\mathrm{cm^{-1}}$, the asymmetric stretching vibration of the $\mathrm{–COO^-}$ bond at 1593 $\mathrm{cm^{-1}}$, and the deformation of the –OH bonds at 1217 $\mathrm{cm^{-1}}$. We can also observe the –C–H and $\mathrm{–CH_3}$ bond stretching present in the molecule at wavenumber 3000 $\mathrm{cm^{-1}}$ and the asymmetric bending deformation of the methyl group at 1452 $\mathrm{cm^{-1}}$. In comparison with the spectrum obtained from the oligomer synthesized from this lactic acid, it is possible to observe the appearance of the –C–O–C– bond, characterized by the signal at wavenumber 1174 $\mathrm{cm^{-1}}$, which shows that the lactic acid is polymerized by means of direct condensation reactions to form a polyester, as well as the disappearance of the $\mathrm{–C–O^-}$ at 1392 $\mathrm{cm^{-1}}$ that

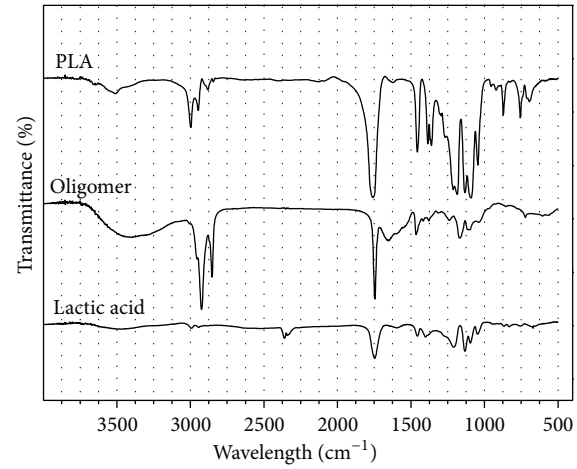

Figure 1: FTIR spectra of lactic acid produced by fermentation with *L. delbrueckii* and PLA synthesized.

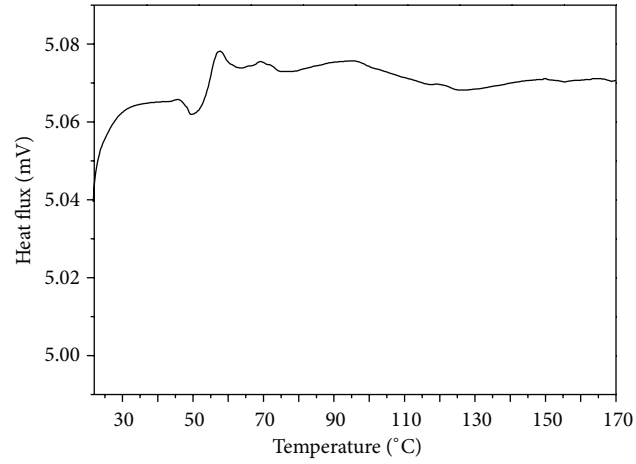

Figure 2: DSC curve of PDLLA synthesized, heated from 20 to 170°C at 2°C $\mathrm{min^{-1}}$.

was present in the lactic acid. In the case of the PDLLA formed from the lactide, the FTIR spectrum clearly shows the asymmetric stretch of the polyester bond formed at wavenumber 1182 $\mathrm{cm^{-1}}$ and the deformation of the –C–O bond at 1091 $\mathrm{cm^{-1}}$.

The intrinsic viscosity of the PLA was 30.028 mL/g. The Mark-Howink K and a constants used were of 0.0549 and 0.639, respectively, values found by Jeantet et al. [31] on studying PLA for packing applications. The molecular weight was calculated from this value using the following equation: $[\eta] = KM^a$, to obtain an Mv of 19,264 g/mol. The presence of impurities that could not be removed by means of the RO process, water absorbed during a poor drying process or before polymerization, and which may be contained originally in the catalyst can affect lactide polymerization leading to a degradation of the polymer and interference during the ring-opening reaction, so preventing the molecular weight of the PLA from increasing. Brás et al. [32] have demonstrated this to be the case, and Kimura et

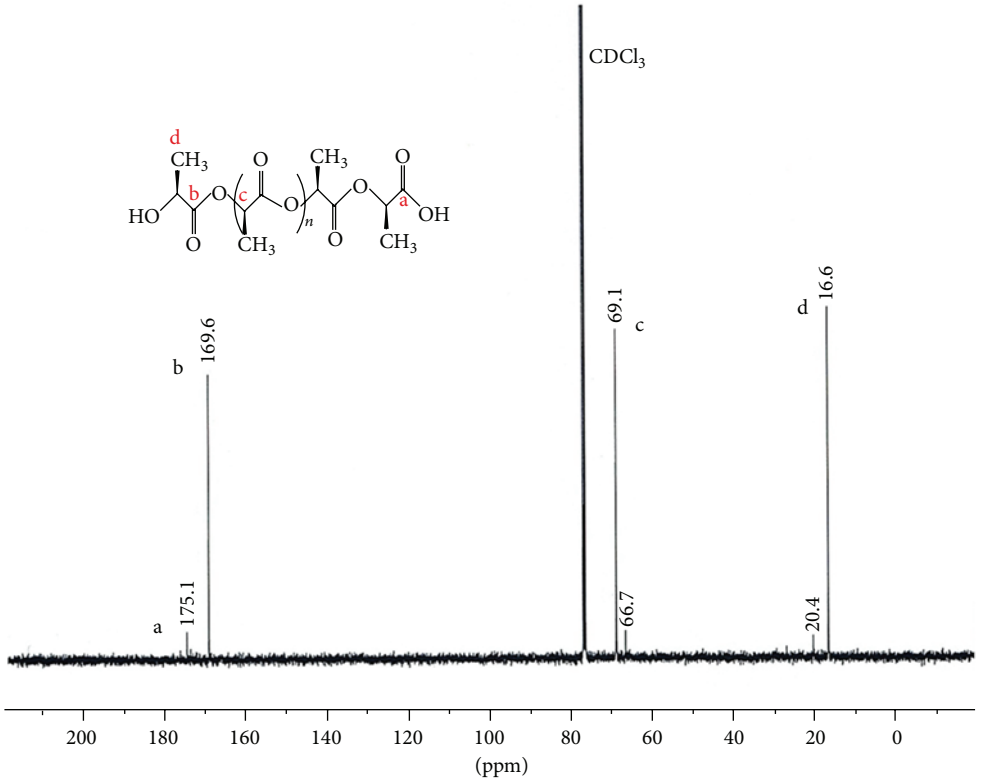

Figure 3: Spectrum ^{13}C-NMR of PDLLA.

al. [33], among others, have shown that the water molecule produces hydrolysis of the polyester causing the bonds in the chains to break, thereby affecting molecular weight.

The glass transition temperature T_g of the PDLLA obtained in this study by ring-opening was 54.34°C. Figure 2 shows that the endothermic phase of the polymer lies between 50 and 58°C as it approaches its T_g. After this value, no changes occur in the material that indicate another material transition, confirming that the synthesized PDDLA is an amorphous polymer with a low molecular weight.

Stolt et al. [34] reported a molecular weight with an Mn number of 3500 g/mol with a T_g of 22°C, whilst Zhao et al. [35] obtained a PDLLA with an Mv of 4,100 g/mol and a T_g of 48.17°C. When the molecular weight of the PLA reached 33 000 g/mol, the T_g reported by Kim and Woo [36] was 59°C. The glass transition temperature increased as the molecular weight increased. The T_g reported in this study lies in the intermediate values compared to those reported in the literature and agrees with its molecular weight.

The ^{13}C-NMR spectrum in Figure 3 allows us to corroborate the chemical structure of the PDLLA, where the signals at δ = 16.6, 69.1, 169.6, and 175.1 ppm correspond to the –CH_3, –CH–, –O–C=O, and –COOH groups, similar to the signals obtained by Chen et al. [37] when characterizing the structure of PLLA when polymerized by direct condensation with titanium(IV) butoxide as a catalyst. This causes racemization reactions in the *L*-lactic acid and signals similar to a PDLLA are obtained for the polymer. On the other hand, the signals match those obtained by Lei et al. [38], who performed a qualitative analysis of the chemical structure of PDLLA via this technique to confirm the results obtained by FTIR and ^{1}H-NMR.

The signals in the ^{1}H-NMR spectrum in Figure 4 corroborate the chemical structure of the PDLLA with the doublet from 1.569 to 1.617 ppm that corresponds to the hydrogen of the –CH_3 group of the polymer body. The signals at 1.49 ppm are the methyl terminals, whilst the quartet observed at 5.20 ppm is characteristic of the hydrogen present in the –CH– of the polymer. The signals from 4.35 to 4.38 are from the –CH– terminals. The hydrogens of the hydroxyl group corresponding to the ends of the polymer chains is observed with the singlet at 3.89 ppm. These signals are very close to those reported by Lei et al. [38] and Konishi et al. [39], both of whom worked with PDLLA. In the first case, polycondensation was performed with a tin chloride catalyst, whilst the Konishi group used metal plates to observe the racemization behavior by determining the signal differences between PLLA and PDLLA.

4. Conclusions

The production of *D,L*-lactic acid in this study was performed by means of lactose fermentation using the *L. delbrueckii* strain with a yield of 0.99 g lactic acid/g lactose in a batch system for 48 h.

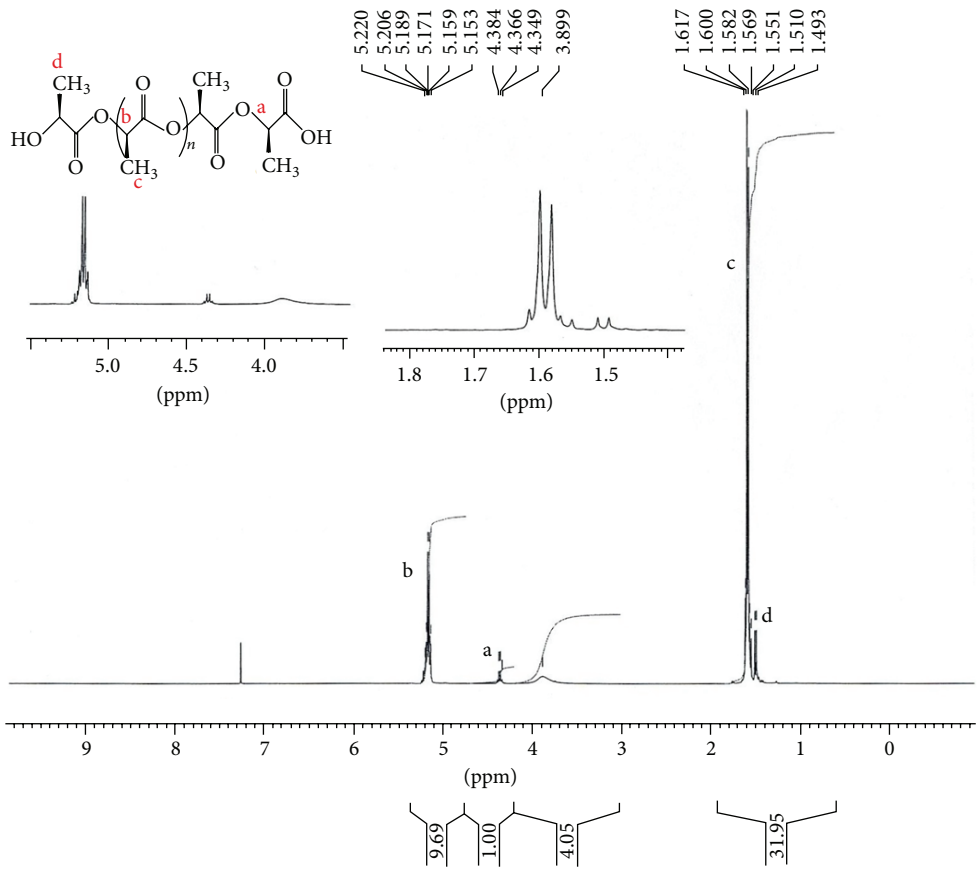

Figure 4: Spectrum ^{1}H-NMR of PDLLA.

The lactic acid concentration in this study was 80.95 g/L, of which 38.23 g/L corresponded to *D*-lactic acid and 42.72 g/L to *L*-lactic acid, producing a translucent product with an oily consistency.

Lactic acid polymerization was performed with ring-opening reactions to obtain PDLLA with a molecular weight Mv of 19,264 g/mol.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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