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Pilot Scale for Production and Purification of Lactic Acid from *Ceratonia siliqua* L. (Carob) Bagasse

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Abstract: The bioconversion of lignocellulose and organic waste bagasse to lactic acid (LA) is an important alternative process requiring valorization as a potentially viable method in the production of pure LA, to be utilized for various purposes. Carob (*Ceratonia siliqua* L.) biomass was used for the production of LA, using a thermophilic *Bacillus coagulans* isolate, cultivated in a batch pilot scale of 35 L fermenters without yeast extract supplementation, and operated for 50 h. During the fermentation process, most of the degradable sugar was consumed within 35 h and resulted in the production of 46.9 g/L LA, with a calculated LA yield of 0.72 g/g sugars and productivity at the log phase of 1.69 g/L/h. The use of LA for different industrial applications requires high purity; therefore, a downstream process (DSP) consisting of different purification stages was used, enabling us to reach up to 99.9% (*w/w*) product purity, which indicates that the process was very effective. The overall almost pure L-LA yield of the DSP was 56%, which indicates that a considerable amount of LA (46%) was lost during the different DSP stages. This is the first study in which carob biomass bagasse has been tested on a pilot scale for LA production, showing the industrial feasibility of the fermentation process.

Keywords: carob bagasse; fermentation; *Bacillus coagulans*; lactic acid; downstream process

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1. Introduction

Carob (*Ceratonia siliqua* L.) belongs to the family Cesalpiniaceae, a subfamily of the family Leguminosae, and is a perennial leguminous, evergreen plant, widely grown in mountain areas of the arid and semi-arid areas of the Mediterranean region, with great economic and environmental potential for ornamental as well as industrial purposes [1,2]. Carob produces edible pods, which are used as fodder for breeding cattle; it has long been considered useful as a source of health products, and as a raw material, used in different food industries, including animal feed, cakes, and yogurt production, as well as for drug delivery purposes [3–5].

The chemical composition of the harvested pods depends on the cultivar, origin, and harvesting time, where its constituents are pulp (90%) and seeds (10%) by weight [6]. Carob pulps are rich in sugars (48–56%) consisting mainly of sucrose, glucose, fructose, and maltose, in addition to 18% cellulose and hemicelluloses, (3–4%) protein, and (0.4–0.8%) lipids [6]. The main sugars are glucose (7–10%), fructose (10–12%), and sucrose (34–42%) [6], used in the production of different types of syrups, where the remaining bagasse, consisting of the lignocellulosic, is mostly discarded as waste. A recent study showed that carob bagasse, in dry matter form, consisted of 27.7% sugars, 19% cellulose, 0.35% hemicellulose, 28.4% lignin, and 9.5% protein [7].

Recently, interest in using carob pods has been increasing because of a cheap source of various fermentation products such as lactic acid (LA), where the highest value of LA 49.34 g/L was obtained from carob syrup enriched with 10 g/L of beef extract [8].

The bioconversion of lignocellulose biomass and waste bagasse to LA is an important resource to be valorized as a method of producing LA to be used in the food industry, cosmetics, pharmaceuticals, and as polylactic acid (PLA), a biodegradable plastic [7,9,10]. The worldwide demand for LA is increasing, with demand expected to reach 1960 kilo-tons by 2025 [11,12].

Carob bagasse was utilized for LA production without pretreatment, using the fermentation process of a separate hydrolysis and co-fermentation (SHCF) process, which showed that there is no need to add yeast extract, and resulted in a high yield of 0.84 g LA/g sugars, and productivity of 2.30 g/L/h [7].

It is well known that the production of PLA needs high optical purity of the produced LA, and the process of purification requires many steps, starting from the pre-treatment of the used biomass, through hydrolysis, and fermentation, up to the downstream process (DSP), in order to obtain high-purity LA [13]. The DSP during the fermentation production process, and purification of LA, is still an important industrial challenge, mainly when the starting biomass includes lignocellulosic biomass and bagasse as the fermentation feedstocks, where, in many cases, it requires physical and chemical pretreatment processes [7,14–16].

Different studies have been conducted on LA DSP, including precipitation, solvent extraction, adsorption, ion exchange, membrane separation processes, and reactive separation systems. Most of these studied processes need specific equipment and involve solvent-recovery costs and high energy consumption [17–19]. Many of the available technologies have not been tested in large-scale fermentation of second-generation LA separation and purification, where the production process is based on the use of lignocellulose or bagasse biomass. Currently, purification of LA is focusing on alternative methods for the purification of LA from crude fermentation broths, especially from lignocellulose biomass where the most promising DSPs are based on the use of ultra- and nanofiltration, electrodialysis, ion-exchange/adsorption, reactive distillation and hybrid short path evaporation [9,18–20]. Some of the recent studies have performed downstream separation of LA from complex fermentation broths [15,21–23].

Based on previous studies at our lab, we decided to use a modified downstream process—using different stages to purify LA—in pilot-scale fermentation [15]. The aim of this study was to produce LA with high purity from carob bagasse, using a pilot scale and DSP based on different stages of filtrations, softening, electrodialysis (ED), decolorization, and cation/anion exchange, combined with vacuum distillation, in order to obtain a high-purity concentrated LA product.

2. Materials and Methods

2.1. Feedstock and Biomass Analysis

The syrup was extracted from carob (*C. siliqua* L.) dried pods collected from Nazareth, the Galilee region, Israel, as previously described [7]. The remaining bagasse biomass was dried in air for a few days, and then in a vacuum oven for 24 h at 50 °C, before being ground, using a corn grinder, and stored at room temperature until required.

The main components—consisting of glucose, disaccharides, and fructose of the different lignocellulosic bagasse—were evaluated, using the laboratory analytical protocol (LAP) developed by the national renewable energy laboratory (NREL), using 100 g of carob bagasse [24].

2.2. Larger-Scale Fermentation for Biomass

The scale-up process was carried out using two bench-scale fermentation (70 L BIOSTAT UD bioreactors, B-Braun Biotech, Germany), each for 50 h, with each 35 L working volume containing carob biomass at 30% DM (10.5 Kg bagasse per fermenter), to produce

LA using the strain *Bacillus coagulans* isolate A107. Carob biomass hydrolysis was carried out for 24 h as previously described [7], and then protein hydrolysis was carried out using 0.0067% (*v/w*) Neutrase 0.8 L and 0.0067% (*v/w*) Flavourzyme 500 L with incubation for 30 min. The protein hydrolysis was conducted based on previous work, which showed that this process enhances the growth rate of the *B. coagulans*. Fermentations were conducted at 52 °C, with stirring at 400 rpm, and the pH was adjusted to 6.0 with 20% (*w/w*) NaOH. Inoculum volume was 6% (*v/v*).

2.3. Sampling Analysis and Lactic Acid Optical Purity

Sampling and analysis were conducted as previously described [7]. Briefly: Samples were taken aseptically, for the analysis of the different sugars, acetic acid, and LA concentrations. The production of sugars, acetic acid, and LA from each fermentation sample were carried out using HPLC (Dionex, Sunnyvale, CA, USA), equipped with a Eurokat H column (300 mm × 8 mm × 10 µm, Knauer, Berlin, Germany). The detection of the different components was carried out using a UV detector.

Lactic acid optical purity analysis was conducted using HPLC (Knauer, Berlin Germany) coupled with a Chiralpak®MA(+) column (50 mm × 4.6 mm × 3 µm, Daicel, Tokyo, Japan), using 2 mM $CuSO_4$ as mobile phase, at a flow rate of 0.8 mL/min. Detection was carried out with a UV detector.

2.4. Downstream Process

The downstream process (DSP) was carried out as described in Figure 1.

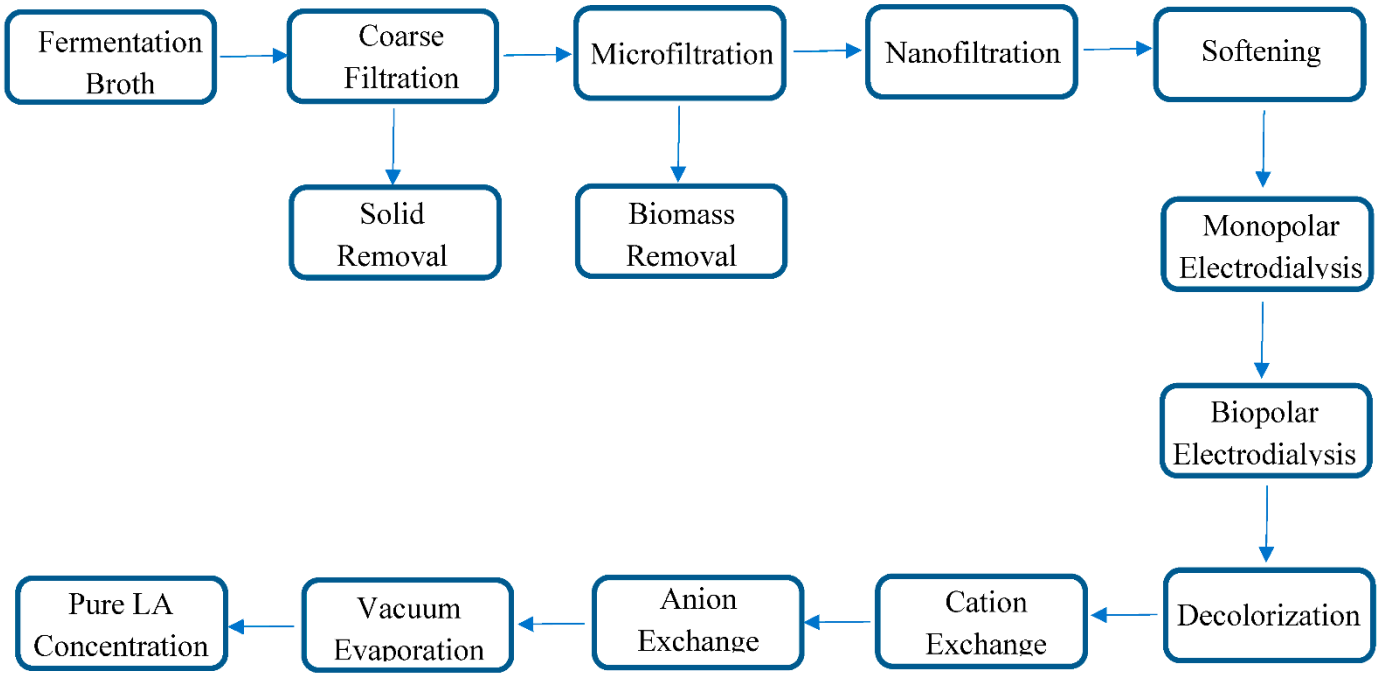

Figure 1. Outlines of the downstream process of lactic acid.

2.5. Filtration and Softening

Initial filtration was conducted using filter bags (Schwegmann Filtrations-Technik GmbH, Grafschaft-Ringen, Germany), with 150 µm pore size. The obtained permeate stream was then subjected to microfiltration (MF) of 0.2 µm pore size (TAMI Industries, Nyons, France) at 1.5 bars, using a filtration device (UFI-Tec GmbH, Oranienburg, Germany). Then a nanofiltration (NF) process was carried out using a 200 Da FILMTEC™ NF membrane NF-2540 Active area 2.6 m^2 (DuPont de Nemours, Wilmington, DE, USA) and filtration device of UFI-Tec GmbH, Oranienburg, Germany. This filtration step was carried

out in order to separate compounds with higher molecular weight, such as proteins, while passing monovalent salts. A large proportion of the divalent ions were retained too.

Bivalent cations' removal (softening) was conducted using PUROLITE S950 acid chelating resin (Purolite, Ratingen, Germany) packed in an expanded bed setting (UIT GmbH, Dresden, Germany). This process is a protective step, used to completely separate divalent metal ions, such as magnesium and calcium, with resins. These would otherwise precipitate in bipolar electrodialysis and damage the membranes.

2.6. Electrodialysis

The monopolar electrodialysis (ED) divided the ionic compounds from the non-ionic compounds and was carried out using FujiFilm Ion Exchange membrane Type 2 CEM (cation-exchange membrane) and AEM (anion exchange membrane) Fujifilm Manufacturing Europe B.V., Tilburg, Netherlands. The ED device used was from Deukum GmbH Frickenhausen, Germany. During this process, ions (sodium lactate), cations, and anions in the concentrate stream, and the other components (sugars, etc.), stay in the diluent stream.

A bipolar ED step then followed, using PC bip (bipolar membrane) PC 100D (anion exchange membrane) PC SK + PC SC (cation exchange membrane) PCCell GmbH, Heusweiler, Germany. During this process, the sodium lactate was split into LA and sodium hydroxide solution. In addition, the other salt components separated into acid and base. As in the monopolar ED, the ions moved in the electric field, passing through the membrane with the appropriate electrical charge. The bipolar membrane is a catalytic intermediate layer, used in order to accelerate the splitting of the water into protons and hydroxide ions. This creates 3 streams: an acid stream, a base stream, and a depleted salt stream. In our process, a free LA stream was obtained after the bipolar ED, together with a NaOH solution. Both EDs were carried out in batch mode, under constant polarity, and at a temperature of 35 °C. The acid stream was used for the subsequent purification steps.

2.7. Decolorization, Cation and Anion Exchange

A strong acid resin, consisting of PUROLITE MN-502 (Purolite Corporation, King of Prussia, PA, USA) filled in a column, (UIT GmbH, Dresden, Germany), was used to remove the color impurities.

Since part of the cations (i.e., sodium) was still in the acid stream after the ED process, we decided to use cation resin RELITE EXC08 (strongly acidic resin), (Resindion S.r.l., Binasco, Italy), filled in a column, (UIT GmbH, Dresden, Germany), to remove it from the stream.

Inorganic acids, in particular, are sometimes present in higher concentrations in the acid stream. These were removed with the anion exchanger using resin Purolite® A133S (weak base anion exchange resin) (Purolite Corporation, USA), and filled in a column (UIT GmbH, Dresden, Germany). For this purpose, an exact calculation of the resin quantity had to be made, otherwise, the LA would have been permanently bound to the ion exchange resin. Weak ions such as lactate are displaced by stronger ions such as chloride or sulfate from the resin.

2.8. Distillation\Evaporation

The filtrate was finally vacuum-evaporated using a vacuum distillation device (Type CR15, Büchi AG, Uster, Switzerland) at 55 °C, −0.99 bar, in order to concentrate the LA. The operation of this process did not change the L-LA to D-LA ratio.

3. Results and Discussion

3.1. Carob Content

The composition of the carob used in these experiments consisted of: protein, sugar, cellulose, hemicellulose, and lignin, with dry matter percentages of: 9.3, 27.7, 19.0, 0.35, and 28.4, respectively. It is known that carob is rich in fiber, with relatively low cellulose and hemicelluloses (18%), high levels of tannins (16–20%), antioxidants, and phenolic

compounds (2–20%), and relatively high levels of proteins (2.7–7.6%) [4]. Our results showed that the combined cellulose and hemicelluloses of carob formed 19.35% of the total biomass, and mainly consisted of cellulose.

3.2. Large-Scale Fermentation

Figure 2a,b shows sugar types and content of carob (30% DM) bagasse after enzymatic hydrolysis, used for LA production, without yeast extract, using SHCF process of two 35 L working-volume-scale fermenters. This resulted in total sugar content of 70.6 g/L, consisting mainly of glucose and fructose, at 38.8 and 28.0 g/L, respectively, where the disaccharides hydrolyzed into glucose and fructose.

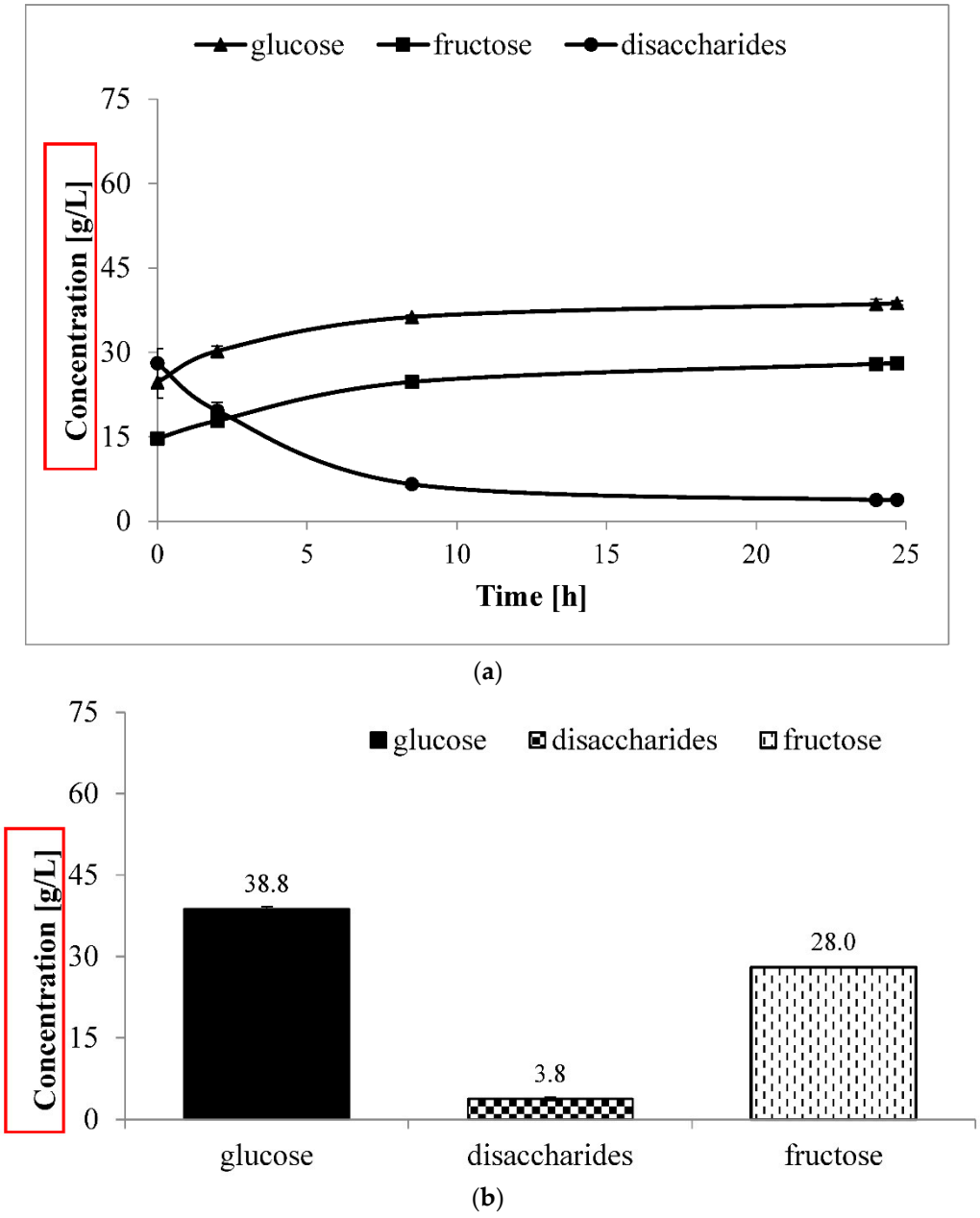

Figure 2. (**a**) Sugar types and content (g/L) as a function of enzymatic hydrolysis time of carob biomass (30% DM) used for LA production during enzymatic hydrolysis using two different 35 L pilot scales. Each data point is the mean ± SD. (**b**) Sugar types and final content (g/L) of carob bagasse (30% DM) were used for LA production during enzymatic hydrolysis using two different 35 L pilot scales. Each bar is the mean ± SD.

In both fermenters, during the 50 h fermentation process, it was noticed that most of the degradable sugar was consumed (Figure 3a), resulting in an average production of 46.9 g/L LA; no acetic acid was produced and the remaining content of unconsumed sugars was 12.70 g/L (Figure 3b).

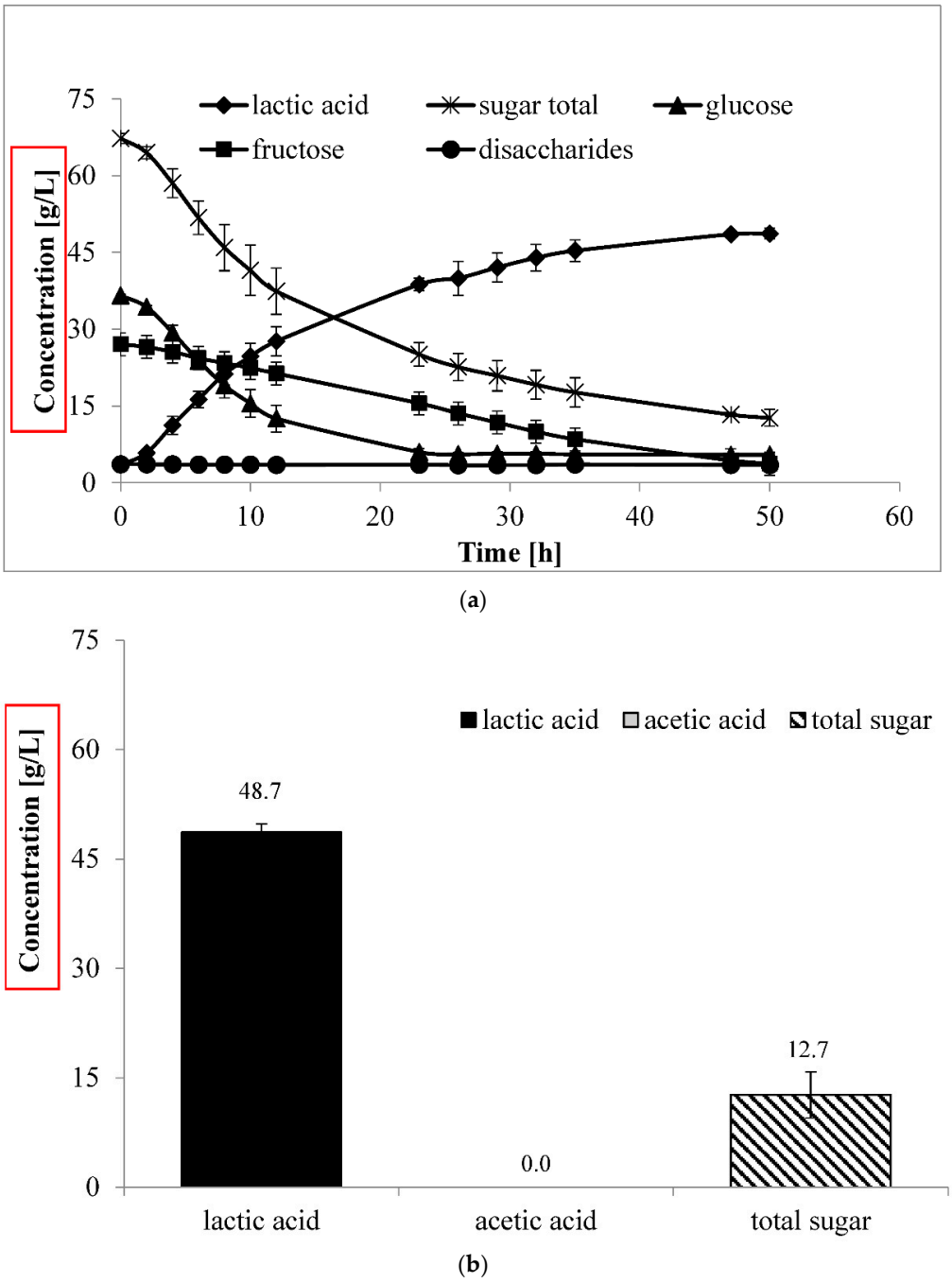

Figure 3. (**a**) *B. coagulans* fermentation carried out on a pilot scale (35 L) using SHCF as the sole carbon and nutrient source. Fermentative production of LA and consumption of glucose, fructose, and disaccharides, with time, during 50 h of fermentation, using 2 pilot scale fermenters. Each data point is the mean ± SD. (**b**) The *B. coagulans* fermentation process was carried out on a pilot scale (35 L) using SHCF as the sole carbon and nutrient source. Fermentative production of LA and lactic acid, acetic acid, and total sugars at the end, after 50 h of fermentation using 2 pilot scale fermenters. Each bar is the mean ± SD.

Fermentation was carried out using a 35 L working-volume fermenter and the profile of the process is shown in Figure 3a. As in the lab scale experiments [7], LA production using isolate A107 showed a lag phase of about 2 h, and the glucose concentration decreased rapidly through the first 12 h of fermentation. In addition to glucose, the consumption of fructose also occurred at a good rate, while the disaccharides' consumption was not noticeable. After 35 h of fermentation, most of the sugars were consumed and LA production reached a maximum concentration of approximately 49 g/L, with a calculated LA yield of 0.72 g/g sugars, and productivity at the log phase of 1.69 g/L/h, acetic acid was 0.0 g/L and the remaining content of unconsumed sugars was 12.7 g/L (Figure 3b).

3.3. Downstream Processing of Lactic Acid

The use of LA for different industrial applications requires high purity. Our results showed low levels of residual sugars and proteins remaining in the medium after the fermentation process. In addition, phosphorus and other ions were still present at high concentrations (Table 1). In our DSP process, we used several steps to remove impurities, in order to separate LA from the rest of the fermentation components. Another group proposed a hybrid integrated membrane separation process, consisting of UF, NF, ion exchange, and vacuum-assisted evaporation. In our study, after the fermentation, a coarse filtration step was used in order to separate larger particles that may damage or block the UF membranes [25]. LA losses of 26% were observed in this step, in addition to a decrease in all the other components of interest: anions, cations, and total nitrogen (determined using Kjeldahl) (Figure 4, Table 1). The permeate stream was subsequently subjected to MF and then an NF; these filtration steps were carried out to separate compounds with higher molecular weight, such as proteins, while allowing monovalent salts to pass. A large proportion of the divalent ions were also retained.

Table 1. Compositional analysis was detected after every step of the downstream process of the fermentation broth, obtained during the pilot scale fermentation process, using the carob bagasse as a feedstock.

	V	LA	PO_4^{3+}	Cl^-	SO_4^{2+}	NO_3^-	Na^+	K^+	Mg^+	Ca^{2+}	NH_4^+
	[L]	[g·L^{-1}]					[mg·L^{-1}]				
End fermentation	50.6	46.4	26.6	273	118	3.89	12,762	1627	152	427	3.07
Filtrate 150 µm	36.2	50.9	66.3	267	119	1.30	12,081	1640	149	436	5.34
Permeate microfiltration	48.25	37.6	47.4	171	69.6	1.41	8765	1147	101	258	8.81
Permeate nanofiltration	64.5	21.9	15.7	124	12.8	1.39	4858	622	16.7	50.7	5.37
Softening	72.1	19.8	18.0	115	21.2	1.92	4701	450	0.42	5.24	2.3
Monopolar electrodialysis concentrate	12.3	94.0	84.7	698	90.1	6.37	22,529	2082	<0.1	3.85	2
Bipolar electrodialysis acid	10.51	102	83.0	815	100	7.14	3047	305	<0.1	1.12	1.5
Decolorization	19.6	53.6	41.9	247	48.1	3.95	0.87	0.16	<0.01	0.15	0.05
Cation exchanger	21.2	48.9	42.1	216	44.9	3.49	0.67	0.15	0.01	0.14	0.13
Anion exchanger	22.0	46.3	14.1	2.39	16.3	0.03	0.67	0.24	0.01	0.14	0.12
Concentrate vacuum distillation	1.14	864	223	69.1	200	0.00	133	6.63	0.25	4.01	1.64

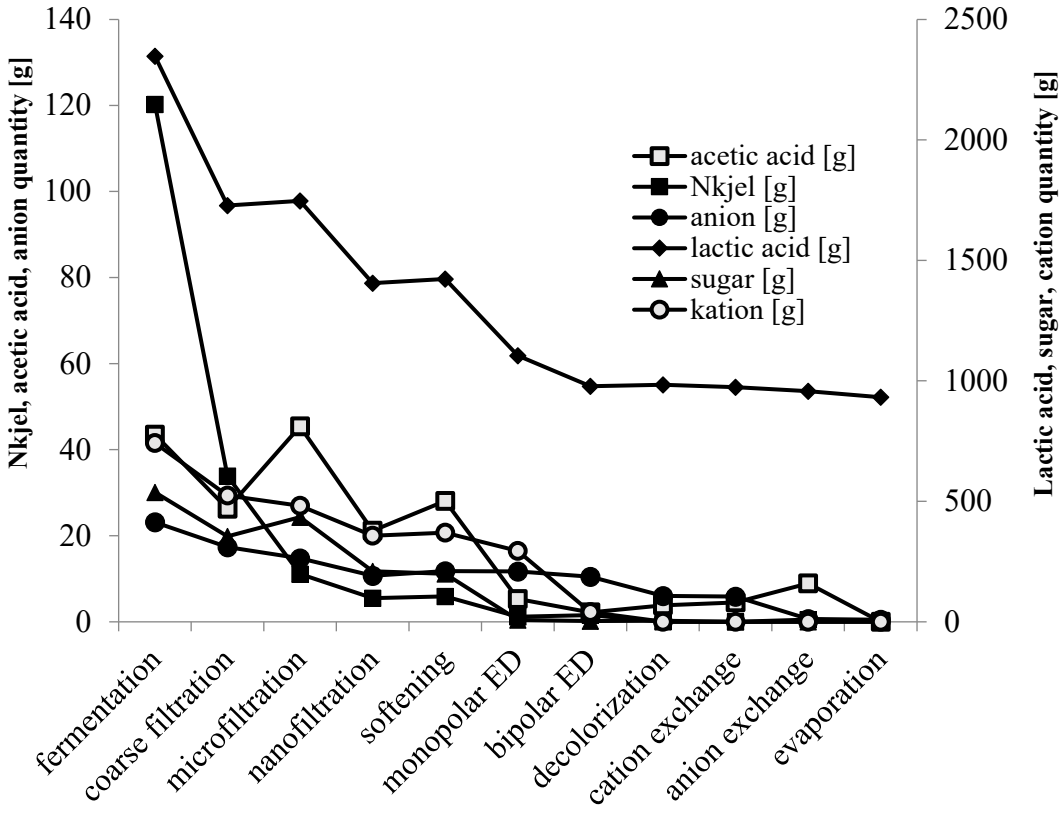

Figure 4. The average compositional analysis of lactic acid, acetic acid, and sugar content following every step of the downstream process in the two pilot scales of the carob bagasse fermentation with 30% solids.

Different technologies have been proposed for the DSP separation and purification of LA from the crude fermentation broths, including ED, which has potential as a promising solution [26–31]. In the current study, monopolar electrodialysis (MED) was investigated after the filtration processes and bipolar electrodialysis (BED) was subsequently employed. Before ED, a softening step was necessary for the removal of divalent ions (mainly Mg^{2+} and Ca^{2+}), which can cause fouling of the ED membranes. As can be seen from Table 1, the concentration of Mg^{2+} and Ca^{2+} after softening was 0.32 ± 0.14 mg/L and 5.4 ± 0.22 mg/L, respectively, with these values corresponding to the removal of 99.7% and 98.7%, respectively. This stream was initially treated with MED, which generated two streams: the concentrate and the diluent. In this work, a volume of 12.8 ± 0.64 L of concentrate stream, containing 86.9 ± 10.04 g/L of sodium lactate, was obtained. In another work using ED, Chen et al., 2016, achieved 90% demineralization from acid whey. The suitability of different DSP options, such as reactive extraction, adsorption, ED, esterification, and reactive distillation, was evaluated, and the authors indicated that the conventional precipitation of calcium lactate, followed by acidification, esterification, and hydrolysis, was the most economical route, although it produced a large amount of gypsum sludge, which needed additional treatment [32]. Additionally, in our work, a considerable reduction in total nitrogen (80%) and in total phosphorus (19% removal) was achieved after this step. The BED step was then conducted, resulting in three streams: acid (10.7 ± 0.24 L), salt (7.1 ± 1.48 L), and base (12.6 ± 0.57 L). We have shown a recovery of 90.4 ± 0.44% of LA, which was observed in the acid stream, whereas only 9.6 ± 0.49 g/L was lost into the base stream, which can be reused in the next fermentation process.

The acid stream was further treated using a decolorization step in order to remove the residual compounds that contributed to the yellowish color of the stream. Cation and anion exchange resins were used before the vacuum evaporation in order to remove the residual ions. By applying chromatography, more than 90% of the monovalent ions were successfully removed. So far, different separation processes have been proposed in different works, including: solvent extraction; membrane separation; ion exchange chromatography; and reactive distillation; aiming to overcome the different drawbacks of the DSP, in order to minimize the LA losses during the process [9].

In our current work, the effective LA downstream resulted in 99.9% (*w/w*) product purity using carob bagasse, which is considered as agricultural waste. Pure LA is a suitable raw material for producing PLA, since the production process requires high optical purity [9,33]. PLA is a very attractive product because it can be produced from renewable resources such as lignocellulose and bagasse feedstock, which is considered a biodegradable product. This has led PLA to gain momentum as an alternative product to petroleum-based plastics. The production of PLA (which is well known as a biodegradable plastic polymer), is based on the use of renewable sources of lignocellulose biomass, to minimize environmental problems caused by synthetic plastic (which is not biodegradable), and is, therefore, now considered a promising and sound approach [9,33]. LA production requires several steps, starting with biomass pre-treatment, through the hydrolysis of the feedstock, followed by the fermentation process, and finishing at the DSP, where some of the LA is lost, with the amount depending on the process [15]. In our case, using carob bagasse, a considerable amount of around 46% of the produced LA was lost during the DSP. Therefore, the DSP process should be optimized during the industrial process in future research, where some of the used stages may be removed or modified. Since the LA market is in constant growth, with great potential to produce various products, and the fact that the final products are able to comply with environmental requirements, such as being green, renewable, and biodegradable, there is much research in this field.

Finally, a 1.1 ± 0.06 L solution containing 862.4 ± 1.7 g/L of LA was obtained, consisting mainly of L-LA with an L-LA/D-LA content of 99.65, which didn't change during DSP (Table 2). High LA purities are of major importance, as already indicated, especially when the production of PLA is the final goal. These processing steps resulted in high LA purity; however, the overall LA yield (from the end of the fermentation until the final distillate solution) was, ca., 56%, meaning that a considerable amount of LA was lost (46%) during the different treatments (Figure 5). The effective LA downstream enabled us to reach 99.9% (*w/w*) product purity, whereas a similar process, used to produce LA from sweet sorghum juice, resulted in 98.9% optical purity [15]. To the authors' knowledge, this is the first study in which carob bagasse has been tested on a pilot scale for L-lactic acid production, showing the industrial feasibility of the process. The obtained high-purity LA from bagasse is a promising approach to converting environmentally polluting biomass, which may lead to materials such as carob being used to produce pure LA, for use in the production of food additives, PLA, and other products within the industrial economy.

Table 2. The amount of the different lactic acid isomers obtained at the end of the downstream process after the evaporation process in both pilot scale fermenters using carob bagasse. Each value is the mean ± SD of two replicates.

D-LA (g/L)	L-LA (g/L)	Total LA (g/L)	L/D (%)
3.2 ± 3.1	859.25 ± 1.34	862.4 ± 1.7	99.65 ± 0.07

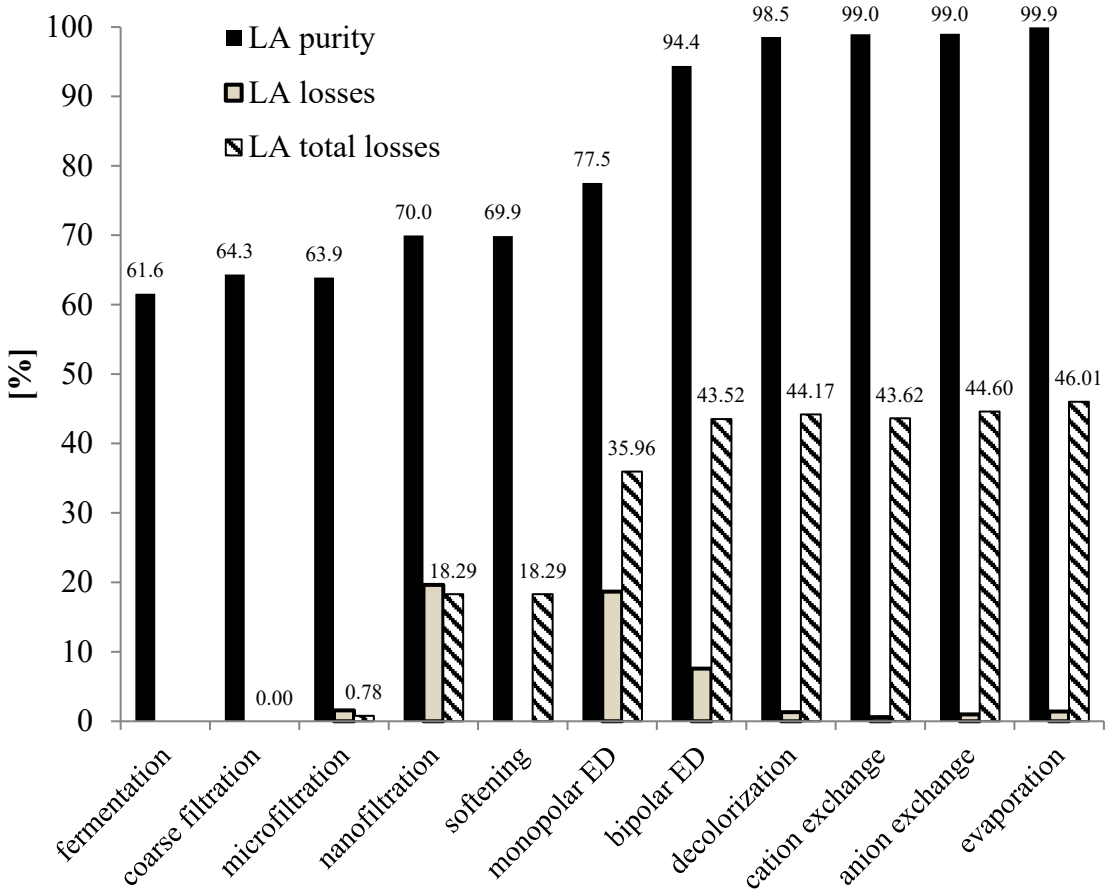

Figure 5. Average purity of lactic acid, accumulative total losses (%) after every step of the downstream process of the two pilot scales of the carob bagasse fermentation with 30% solids.

4. Conclusions

The results obtained in this work indicated that carob is a promising substrate for L-lactic acid production. Using two pilot scales of 35 L fermenters, operated for 50 h each, the fermentation process showed that most of the degradable sugar was consumed, and resulted in an average production of 46.9 g/L LA, with a calculated LA yield of 0.72 g/g sugars, and productivity at the log phase of 1.69 g/L/h. The effective LA downstream enabled us to reach 99.9% (*w/w*) product purity, which indicates that the purification stage is based on the integrated processes of coarse filtration, microfiltration, nanofiltration, softening, mono/bipolar ED decolorization, cation/anion exchange, and vacuum evaporation. The process was very effective in obtaining high purity L-LA, which may be used in industrial applications, such as PLA production, or as food additives. However, the overall LA yield (from the end of the fermentation until the final distillate solution) was 56%, meaning that a considerable amount of LA was lost (46%) during the different separation-stage treatments, which indicates that improvement of the current DSP is still required in order to be economically feasible.

Author Contributions: H.A. and J.V. developed the idea of the conceptual work; J.V. supervised the conducted work; H.N.A.T. was responsible for collecting and preparation of the Carob biomass; R.S. was responsible for, and conducted, the separation process; H.A. was responsible for conducting the literature review and writing the first draft. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

LA	Lactic acid
DSP	Downstream process
PLA	Polylactic acid
SHCF	Separate hydrolysis and co-fermentation
ED	Electrodialysis
LAP	Laboratory analytical protocol
NREL	National renewable energy laboratory
HPLC	High-Performance Liquid Chromatography
MF	Microfiltration
NF	Nanofiltration
UF	Ultrafiltration
CEM	Cation-exchange membrane
AEM	Anion exchange membrane
DM	Dry matter
Nkjel	Nitrogen Kjeldahl (g)
MED	Monopolar electrodialysis
BED	Bipolar electrodialysis

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