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Proceeding Paper

Evaluation of Agro-Industrial Carbon and Energy Sources for *Lactobacillus plantarum* M8 Growth †

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Abstract: Lactic acid is a compound used industrially due to its properties. There are two methods for its production: chemical synthesis and microbial fermentation. In microbial fermentation, food industry waste can be used as a substrate, providing a route towards achieving a circular economy. Thus, this study evaluated different substrates for *Lactobacillus plantarum* growth, a lactic acid producer, such as molasses, whey, glucose, and saccharose, either alone or supplemented with additional nutrients. Bacterial growth parameters were assessed using OD_{620} measurement. It was shown that whey supplemented with yeast extract supported the best growth, allowing a $\mu_{max} = 0.63\ h^{-1}$.

Keywords: lactic acid; fermentation; *Lactobacillus*; whey; molasses

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1. Introduction

Lactic acid (LA), also known as 2-hydroxypropionic acid (CAS No. 50-21-5), is an organic acid that has been used in food, pharmaceutical, cosmetic, and chemical industries due to its properties as a pH regulator and also as a flavorant, an acidulant, and a preservative. It is also used as an intravascular mineral solution. Currently, the greatest interest in lactic acid is due to it being the precursor of polylactic acid, a biopolymer of great interest today because of its use in bioplastic production [1,2].

Lactic acid can be produced in two ways, with chemical synthesis from petrochemical substrates, and with microbial fermentation, using residues from the food industry as substrates [2,3]. The production of lactic acid using chemical synthesis, in addition to having negative consequences for the environment, has the disadvantage of producing a racemic mixture of (D) and (L) isomers of lactic acid, which makes this method less desirable for industrial use. However, the production of lactic acid through microbial fermentation offers the possibility of obtaining lactic acid with (L) or (D) conformation. The importance of lactic acid conformation (L or D) depends on the industry in which it will be used. In the food and pharmaceutical industry, the L conformation is preferred because it is easily metabolized by humans [3,4]. Other advantages of the production of lactic acid using fermentation are lower costs of substrates, low operating temperatures, and low energy consumption [3].

Lactic acid fermentation is carried out by different microorganisms, including yeasts (*Saccharomyces cerevisiae*, *Candida glycerinogenes*), filamentous fungi (*Aspergillus niger*), and Gram-positive bacteria, including lactic acid bacteria (*Lactobacillus* sp., *Bacillus* sp., and *Enterococcus* sp.) [5–9].

Renewable sources such as starch, lignocellulosic biomass, microalgae, glycerol, and agricultural waste have been proposed as substrates for lactic acid production. The latter substrate has several advantages because it contributes towards achieving a circular economy, that is, the biotransformation of waste into a value-added product [10–14].

To select the best carbon and energy source for lactic acid fermentation, in this study, the growth kinetics of *Lactobacillus plantarum*, using four different substrates obtained from food industry, were evaluated.

2. Materials and Methods

2.1. Activation of the Lactobacillus plantarum Strain on an Erlenmeyer Scale

A stock of the *Lactobacillus plantarum* M8, kindly provided by MSc Yadira Parra from the Department of Biotechnology of the National University of Asunción, was used. In two test tubes, 5 mL of MRS medium was added. Next, 100 µL of its glycerol stock was added to each tube. The tubes were then incubated at 37 °C for 48 h. From the cultures obtained, stocks were prepared in 30% glycerol and were stored at −80 °C until use.

2.2. Analysis of the Growth Kinetics of Lactobacillus plantarum M8 in Different Culture Conditions

In order to determine the best growth condition for *L. plantarum* M8, batch cultures were performed using different carbon sources: food-grade saccharose (7% *w*/*v*), glucose (7% *w*/*v*), sugarcane molasses (7% *v*/*v*), and whey. Molasses and whey were pretreated prior to the growth kinetics test, as described below. Table 1 describes the different culture conditions used in the selected substrates.

Table 1. Substrates evaluated for *Lactobacillus plantarum* M8 growth.

Substrates	Proportion
Glucose	7% (*m*/*v*)
Glucose with Yeast Extract	Glucose 7%; 10 g/L yeast extract
Glucose with Meat Peptone	Glucose 7%; 20 g/L meat peptone
Saccharose	7% (*m*/*v*)
Saccharose with Yeast Extract	Sucrose 7%; 10 g/L yeast extract
Saccharose with Meat Peptone	7% sucrose; 20 g/L meat peptone
Molasses	7% (*v*/*v*)
Molasses with Yeast Extract	Molasses 7%; 10 g/L yeast extract
Molasses with Beef Peptone	Molasses 7%; 20 g/L meat peptone
Whey	Clarified whey
Supplemented Whey	$MgSO_4$ 0.05 g/L; $(NH_4)_2HPO_4$ 2.5 g/L; $MnSO_4$ 0.005 g/L
Whey with Meat Peptone	20 g/L meat peptone
Whey with Yeast Extract	10 g/L yeast extract
Whey Supplemented with Meat Peptone	$MgSO_4$ 0.05 g/L; $(NH_4)_2HPO_4$ 2.5 g/L; $MnSO_4$ 0.005 g/L; 20 g/L meat peptone
Whey Supplemented with Yeast Extract	$MgSO_4$ 0.05 g/L; $(NH_4)_2HPO_4$ 2.5 g/L; $MnSO_4$ 0.005 g/L; 10 g/L yeast extract

2.3. Diluted Molasses Preparation

Under sterile conditions, 25 mL molasses were diluted to 7% (*v*/*v*) in sterile distilled water (the molasses was not sterilized) in 50 mL Falcon tubes. The dilutions were centrifuged at 9000 rpm for 5 min, and the supernatant was recovered.

2.4. Whey Pretreatment

Clarification pretreatment using $CaCl_2$ [13]: A 4% (*w*/*v*) $CaCl_2$ solution was added to 1000 mL of whey to obtain a concentration of 0.02% (*v*/*v*) and autoclaved under standard conditions for 15 min. This mixture was then centrifuged at 9000 rpm for 5 min, and the supernatant was recovered. Small colloids that remained were filtered out via filter paper. The clarified whey was then sterilized and stored at 4 °C until use.

2.5. Activation of the Lactobacillus plantarum M8

From *Lactobacillus plantarum* M8 glycerol stocks, 200 µL was taken and transferred to a 5 mL MRS-Broth medium in triplicate, incubated at 37 °C for 19 h. Subsequently, 10 mL of the previously activated strains were inoculated into 200 mL of fresh MRS-Broth medium, and cultured at 37 °C, at 150 rpm. After 28 h, the optical density was determined, and the calculation was performed to start the experimental cultures with an optical density of 0.1.

2.6. Lactobacillus plantarum M8 Growth Kinetics under Different Carbon Sources

From the solutions prepared (saccharose, glucose, molasses, and whey) and the MRS-Broth medium, which was used as control, a 1 mL aliquot was taken and inoculated with the activated strain of *L. plantarum*. Then, 200 µL of each inoculum was transferred to a 96-well plate, in triplicate. Culture medium without cells were used as blanks. Each culture's growth was monitored via optical density measurement at 620 nm (OD620 nm) for 24 h, via plate reader at 37 °C (Multiskan, Thermo Fisher Scientific, Waltham, MA, USA), with pulse shakes before each reading, which was automatically performed every 30 min, along with each reading. Optical density is an indirect measurement of microbial growth, typically used in fermentation assays. As microbes proliferate, the sample's optical density increases linearly until a certain value, usually 0.9, after which samples must be diluted so that the linearity of optical density continues [15]. In our experiments, linearity was maintained with corrections performed automatically by the plate reader.

3. Results and Discussion

3.1. Growth Curves and Biomass Concentration

The data obtained from the growth kinetics of *L. plantarum* M8 using different substrates were analyzed. Figure 1 shows the growth curve in the different glucose conditions, obtaining higher OD620 nm in glucose with meat peptone, followed by glucose with yeast extract. Glucose without supplementation did not support growth, as expected due to the lack of nutrients. Specifically, this may be due to the lack of nitrogen sources and other micro- and macronutrients.

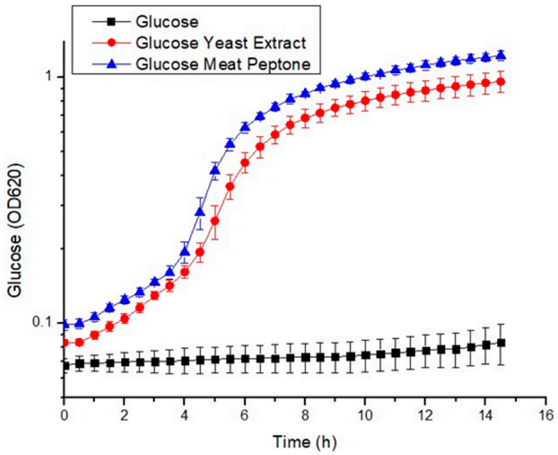

Figure 1. Growth kinetics of *L. plantarum* M8 under different glucose supplementation conditions.

With respect to sucrose, Figure 2 shows that saccharose supplemented with meat peptone and yeast extract were similar. However, the meat peptone's culture started the exponential growth phase approximately 1 h earlier.

Along with glucose and saccharose, molasses, which is an agro-industrial waste, was also evaluated as a substrate. For this evaluation, molasses was diluted to 7% (v/v) prior to supplementation, as described in Materials and Methods. Figure 3 shows *L. plantarum* M8 growth under different molasses supplementation conditions. Molasses without supplementation presented a lower growth than the other two conditions, but a higher growth compared to the growth obtained with glucose and sucrose, both without

supplementation. This may be due to the presence of other limiting elements in molasses, including nitrogen sources and other nutrients. On the other hand, molasses with yeast extract, and molasses with meat peptone, produced similar optical densities. This might indicate the presence of the same limiting elements, and the potential for a higher growth under controlled fermentation conditions.

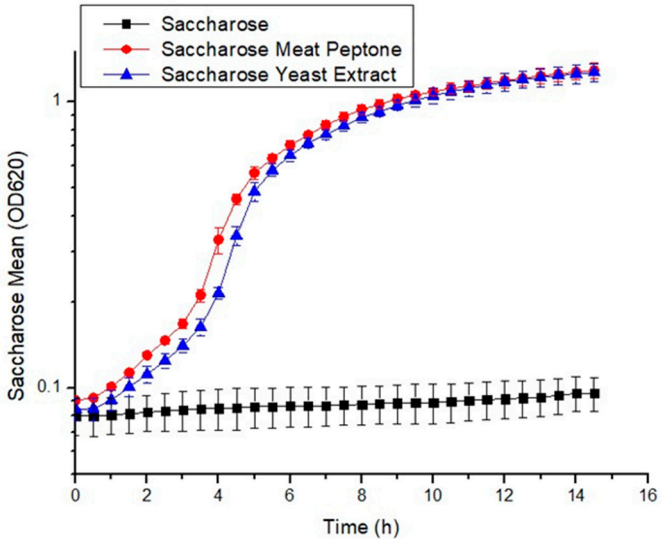

Figure 2. Growth kinetics of *L. plantarum* M8 under different saccharose supplementation conditions.

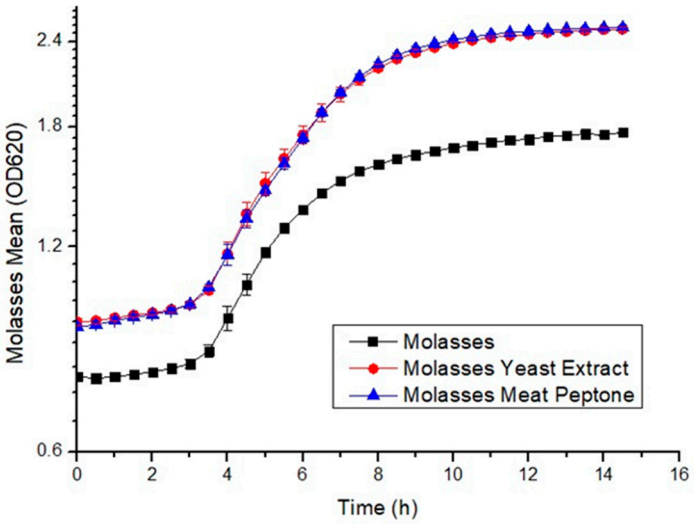

Figure 3. Growth kinetics of *L. plantarum* under different molasses conditions.

Whey, which is another agro-industrial waste, was also evaluated. This substrate was subjected to a clarification process prior to its use, as described in Materials and Methods. Figure 4 shows the growth curves of *L. plantarum* M8 under different whey conditions. The condition containing yeast extract presented the highest final OD620 nm. Neither whey supplemented with salts (red symbols) nor with salts and yeast extract (green symbols) supported microbial growth, as inferred from the obtained flat curves and the formation of precipitates (i.e., high initial OD620).

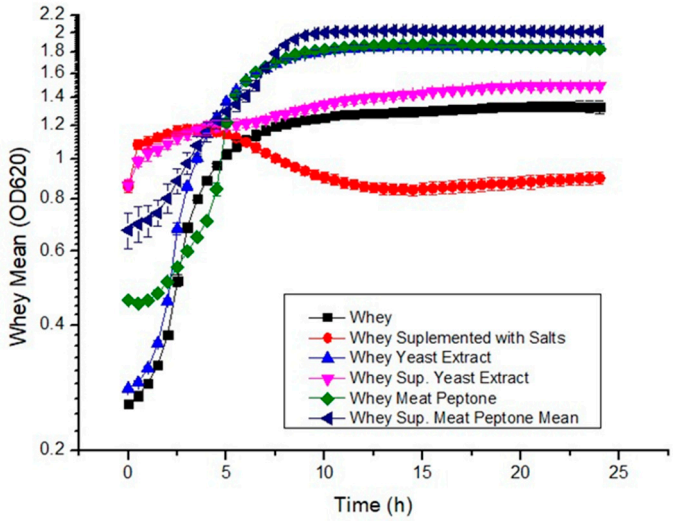

Figure 4. Growth kinetics of *L. plantarum* under different whey conditions.

3.2. Maximum Specific Growth Grates (μ_{Max}) Calculations

The results obtained from the growth kinetics of *L. plantarum* were linearized in order to calculate the maximum specific growth rate (μ_{max}). Table 2 shows the μ_{max} values obtained in the different culture conditions.

Table 2. Maximum growth rate of *L. plantarum*.

Condition	μ_{max} (h^{-1})
MRS	0.67
Molasses	0.2246
Molasses with Beef Peptone	0.2326
Molasses with Yeast Extract	0.26
Saccharose	0
Sucrose with Meat Peptone	0.2519
Sucrose with Yeast Extract	0.7258
Glucose	0
Glucose with Meat Peptone	0.7714
Glucose with Yeast Extract	0.216
Whey	0.59
Whey Supplemented with Salts	0.027
Whey with Yeast Extract	0.63
Whey with Meat Peptone	0.167
Supplemented Whey + Yeast Extract	0.046
Supplemented Whey + Meat Peptone	0.193
MRS Broth	0.65

The μ_{max} obtained in the MRS medium was 0.67 h^{-1}, and this was our study control. The condition that presented the best growth was whey with yeast extract, showing a μ_{max} = 0.63 h^{-1}, followed by whey without supplementation (μ_{max} = 0.59 h^{-1}). With molasses, growth rates were slower compared to the other conditions, although they supported a high final OD6020. Between glucose and sucrose conditions, glucose supplemented with meat peptone had the highest growth rate at μ_{max} = 0.7714 h^{-1}.

Overall, these results suggest that the best growth conditions for *L. plantarum* M8 are whey with yeast extract and unsupplemented whey. These results coincide with those reported by other authors, where both unsupplemented and supplemented whey also present higher lactic acid concentration, volumetric productivity, and yield during fermentation [14,16,17]. In this case, growth is a good proxy for lactic acid production, because this organic acid drives ATP generation and thus growth in lactic acid bacteria.

4. Conclusions

Lactic acid generation using microbial cultures has a potential for supplying this commodity for polylactic acid production or bioplastic production. Considering that the carbon and energy sources compatible with lactic acid bacteria (LAB) growth can be agricultural byproducts, both the bioplastic itself and the process for generating its molecular scaffold are compatible with sustainable industrial practices, including achieving a circular economy. Understanding LAB growth kinetics under these conditions will allow us to achieve better process development and the subsequent optimization of lactic acid production, in terms of yield, concentration, and productivity.

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