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ORIGINAL ARTICLE

Valorization of glycerol from biodiesel industries as a renewable substrate for co-producing probiotic bacteria biomass and acetic acid

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Abstract Glycerol, a byproduct from oil-based biodiesel industries, has been used as carbon and energy sources for growing *Lactobacillus* strains with probiotic characteristics. For such a purpose, crude glycerol (70.6 %) was subjected to a pretreatment with different inorganic acids to remove soap, free fatty acids, and other impurities. The best performance was attained with phosphoric acid at pH 4.0, thus resulting in treated samples containing glycerol whose levels were higher than 96 wt%. The treated glycerol was used to formulate the culture medium and assess the ability of 15 *Lactobacillus* strains to assimilate it. High cell yields (0.34, 0.28, and 0.25 g cell g^{-1} glycerol) were achieved by *Lactobacillus delbrueckii* UFV-H2b20, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus plantarum* ATCC 8014 strains, respectively. Kinetic profiles in medium containing potassium phosphate buffer solution (pH 6.0) showed similar growth (yields ranging from 0.29–0.31 g g^{-1}) and acetic acid production (yields ranging from 0.33–0.34 g g^{-1}) for the selected lactobacilli bacteria. These results reveal that biodiesel-derived glycerol represents a potential substrate for growing probiotic strains, thence obtaining value-added products.

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1 Introduction

Biodiesel is a renewable fuel produced by the transesterification of vegetable oils or animal fat with alcohol (methanol or ethanol) which has recently aroused substantial interest due to its contribution to petroleum-based diesel global dependence reduction. Nowadays, a large expansion of biodiesel industries has been observed, which is estimated to reach a global production of over 40 billion liters in the next decade [1]. Its process generates approximately 10 wt% of glycerol [2] and its production cost varies inversely and linearly, with fluctuations in the market value of glycerol [3].

The amount of glycerol derived from biodiesel industries shows an exponential growth, and its valorization is critical to increase their economic viability and environmental sustainability [4]. Typically, after an onerous distillation process, glycerol is used as feedstock for producing value-added chemicals to be used as additives by food, pharmaceutical, cosmetic, and paper industries. However, small and medium-size industries cannot afford the highs cost of glycerol purification, and consequently, large amounts of glycerol are being accumulated in the environment [5]. Therefore, it is important to develop new and innovative strategies for using crude glycerol.

Glycerol utilization as raw material for obtaining different molecules has received several research groups' assiduous attention due to its abundance and low commercial value (lower than US$ 0.20 per lb) [6]. It is a key molecule for biological processes and feasible to be used as carbon and

energy source by many microorganisms, including yeast, fungi, and bacteria [7–10]. In this regard, its availability associated with low-cost stimulates its utilization as a potential source of carbon for obtaining a variety of bioproducts through biotechnological processes, such as microbial biomass, organic acid, aldehyde, ethanol, protein, and lipid generation [2, 9–11]. Therefore, the glycerol biorefinery concept should be exploited for obtaining high-quality products of industrial value by biotechnological processes with the aim of improving the feasibility and environmental sustainability of biodiesel industries.

A number of chemical elements are present in biodiesel-derived glycerol, including potassium, nitrogen, and phosphorous which can be used as a nutritional resource for different microorganism species [12, 13]. Nonetheless, crude glycerol could also contain salts, toxic metallic ions that are mainly derived from metal vessel reactors and other impurities associated with the raw material, such as fatty acids, soap, and alcohols that can negatively affect microorganism growth [14–17]. Venkataramanan et al. [18] reported that the growth and metabolism of *Clostridium pasteurianum* in a medium containing glycerol is slightly inhibited by the presence of oleic acid, and completely inhibited by linoleic acid during anaerobic cultivation. The removal of soap, among other impurities, improves the fermentability of biodiesel-derived glycerol by some microorganisms, whose consumption rate is similar to that observed with pure glycerol [17, 18]. Therefore, it is fundamental to determine the chemical composition of glycerol by considering the effects of some impurities on its applicability and valorization in biotechnological processes.

Studies on many bacterial species glycerol metabolism have shown that glycerol dissimilation can occur through oxidative and reductive pathways [2, 19–21]. Under aerobic conditions, glycerol is converted into dihydroxyacetone or glycerol-3-phosphate by glycerol dehydrogenase (EC 1.1.1.6) and glycerol kinase enzymes (EC 2.7.1.30), respectively [19]. Under anaerobic conditions, glycerol is dehydrated by glycerol dehydratase (EC 4.2.1.30) to 3-hydroxypropionaldehyde, which is subsequently reduced to 1.3-propanediol by the 1.3-propanediol oxidoreductase enzyme (EC 1.1.1.202) [7, 19–21]. Pure glycerol utilization in bacterial strains and yeasts has been reported as a precursor for acrolein, reuterin, 1.3-propanediol, acetate, and other compounds [18–24].

Although few reports in literature describe the utilization of glycerol as a sole substrate by *Lactobacillus* strains, several works have been already published on the feasibility of using it as co-substrate in a medium containing glucose for biphasic growth of *Lactobacillus* and other species of microorganisms, in which its consumption took place after glucose exhaustion [19, 21]. Such results suggested that the co-fermentation process increases the rate of glycerol assimilation, biomass concentration, and biomolecules production [25, 26]. It seems that the metabolism of sugar co-substrates increases the formation of intermediate metabolites (e.g., pyruvate), which is channeled to NADH production, thus promoting greater glycerol utilization [19, 25, 26]. Therefore, it is worth assessing new potential *Lactobacillus* strains that are able to metabolize glycerol as carbon and energy source for obtaining biomass and biomolecules.

This study is aimed at characterizing and evaluating different treatments of crude glycerol derived from soybean oil-based biodiesel production, and selecting probiotic *Lactobacillus* strains with the ability to grow and produce acetic acid in a medium containing glycerol.

2 Material and methods

2.1 Glycerol

Crude glycerol obtained as byproduct derived from soybean oil-based biodiesel production was kindly provided by Bioverde Biocombustíveis S.A industries (Taubaté - São Paulo - Brazil), having in its composition glycerol 70.6 % ($w\ w^{-1}$) and impurities 29.4 % ($w\ w^{-1}$) mainly soap, salts, water, alkalis, volatile compounds (methanol), and fatty acids, according to the chemical report provided by the company.

2.2 Crude glycerol treatment

The crude glycerol obtained directly from transesterification process (using soybean oil and methanol as feedstock) was denominated glycerol grade G8 considering the original pH 8.0. This glycerol was acidified to neutralize the excess of potassium hydroxide (KOH) used as catalyst and for separating soaps, found mainly under the form of potassium linoleate and potassium oleate. The effectiveness of three different concentrated acids (H_2SO_4, 90 % ($w\ w^{-1}$); H_3PO_4 85 % ($w\ w^{-1}$); and HCl 37 % ($w\ w^{-1}$)) to remove impurities contained in the crude glycerol were evaluated independently. A 500-mL Becker flask containing 250-mL of crude glycerol was heated at 75 °C under constant stirring (200 rpm) for 30 min to reduce the viscosity and evaporate methanol residues. At the same time, 0.1 mL concentrated acid was added progressively to the crude glycerol to adjust the pH to 7.0, 6.0, 5.0, 4.0, and 2.0 for obtaining a glycerol denominated grade G_7, G_6, G_5, G_4, and G_2, respectively. After reducing viscosity and having biphasic formation, the pretreated glycerol was kept overnight in a separating funnel, followed by decantation and separation of the insoluble compounds from the glycerol phase. The treated glycerol was used for the culture media formulation.

2.3 Microorganisms and growth conditions

Fifteen bacterial strains were selected to evaluate their abilities to assimilate glycerol. *Lactobacillus* LAC01, LAC04, LAC06, LAC07, LAC09, LAC19, LAC23, LAC30,

LAC38, LAC40, LAC PCI, and LAC PC2 were previously isolated from human fecal samples at Geraldo Di Biase University Center (Volta Redonda, RJ, Brazil). The *Lactobacillus delbrueckii* UFV- H2b20 strain was obtained from Department of Food Technology of Federal University of Viçosa (Viçosa, MG, Brazil); *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus acidophilus* ATCC 4356 were obtained from the Tropical Culture Collection of Andre Tosselo Foundation (Campinas, SP, Brazil). These strains were maintained at 4 °C in de Man-Rogosa-Sharpe (MRS) slant agar. The *Lactobacillus* cells were activated by transferring a loopful of the stock culture to a test tube containing 5 mL of sterile and modified MRS broth composed by yeast extract (5 g L^{-1}), peptone (10 g L^{-1}), meat extract (10 g L^{-1}), Tween 80 (0.05 g L^{-1}), magnesium sulfate (0.1 g L^{-1}), and manganese sulfate (0.05 g L^{-1}), in which the glucose was replaced by treated glycerol (10 g L^{-1}). The cultures were inoculated in a 125-mL Erlenmeyer flask containing 50 mL of the same medium composition, followed by incubation at 37 °C to reach the growth exponential phase. The *Lactobacillus* strains' ability for using glycerol as carbon source was evaluated in a 125-mL Erlenmeyer flask, containing 50 mL of modified MRS broth with glycerol at 20 g L^{-1} (pH 6.0, unbuffered media). All the strains were individually inoculated into the media in sufficient amount to achieve an initial cell concentration of 0.05 g L^{-1}. The flasks were incubated under microaerobic condition (without agitation) at 37 °C for 24 h.

A kinetic study was carried out using the selected probiotic strains, which showed the highest cell concentration during the screening tests. The experiments were carried out in unbuffered MRS medium containing treated glycerol 25 g L^{-1} and in the same medium buffered with potassium phosphate buffer (0.2 M) at pH 6.0. All the experiments were carried out, at least, in duplicate. Replicates differed by less than 5 %.

2.4 Analytical methods

2.4.1 FTIR-analysis

The removal of soap and free fatty acids from glycerol after treatment with different inorganic acids was determined by Fourier transform infrared spectroscopy (FTIR). FTIR spectra were obtained in a Perkin Elmer Spectrum GX FTIR spectrometer (USA) equipped with a highly attenuated total reflection (HATR) accessory with a resolution of 4 cm^{-1} and ZnSe crystal. The absorption spectrum was obtained by 50 coaveraged scans and recorded at range of 4000 to 400 cm^{-1}. A thin film of each sample was spread directly on the ZnSe crystal for measurement. The spectral data were analyzed by Von Minelt—KnowItAll® Informatics System—and interpreted by examining the data from literature, taking into consideration the infrared absorptions for pure glycerol, fatty acids, and esters [27, 28].

2.4.2 Elemental composition

The analysis of elements such as Ni, Cu, Fe, Cd, Pb, As, Al, Ca, Mg, Na, and K present in the treated glycerol samples were performed in an atomic absorption spectrometer (AAS) (Perkin Elmer; Model: AAnalyst 800) equipped with modules for atomization by flame and graphite furnace. The values were obtained at the range of 185 and 870 nm with a diffraction grating of 1800 lines mm^{-1} using a solid state detector and background correction when used in the flame module. The metallic elements were analyzed by flame atomic absorption spectrometry (FAAS), except for As, Cd, and Pb that used electrothermal atomization (ET AAS), according to the Standard Method 3000—Metals [29]. For metals, samples were subjected to digestion using a strong acid mixture composed of HCl and HNO_3 (4:1 $v\ v^{-1}$). The total phosphorous and organic nitrogen were determined according to the standard methods for water examination [29].

2.4.3 Cell concentration

The cell concentration was determined by optical density (OD) at 600 nm, in spectrophotometer (Beckman DU 640B, Fullerton, U.S.A.). The OD values were correlated to the cell concentration (g L^{-1}) by means of a calibration curve previously established for each bacteria strain. *Lactobacillus* strains were cultivated in MRS agar by pour plate technique to determinate the total viable cell during cultivation. The media sample was centrifuged at 5000 × *g* for 15 min and the supernatant was utilized to analyze the substrate consumption.

2.4.4 Glycerol and acetic acid concentration

Glycerol and acetic acid concentration were determined by high performance liquid chromatographic (HPLC) (Waters, Milford, USA) equipped with a refractive index (RI) detector and Bio-Rad HPX-87-H (300 × 7.8 mm) column at 45 °C, using 5 mM H_2SO_4 as the mobile phase, flow rate of 0.6 mL min^{-1}, and sample volume of 20 μl. All samples were previously filtered through 0.22-μm membrane filters (Millipore) prior to analysis.

2.4.5 Bioprocess parameters

The bioprocess parameters of $Y_{X/S}$ (g g^{-1}, cell-glycerol yield), Q_X (g L^{-1} h^{-1}, cell productivity), cell concentration (g L^{-1}), and glycerol consumption (%) were experimentally determined. The cell (X) yield was calculated by $Y_{X/S} = \Delta_X/\Delta_S$ (g g^{-1}) and the volumetric productivity in biomass was expressed by $Q_X = \Delta_X/\Delta t$ (g L^{-1} h^1), in which: $\Delta X = X_f - X_o$ (X_f = final biomass concentration and X_o = initial biomass concentration), $\Delta S = S_o - S_f$ (S_o = initial glycerol concentration and S_f = final glycerol concentration), and $\Delta t = t_f - t_o$ (cultivation

time). The specific growth rate (μ, h^{-1}) was calculated by linear regression of the ln (A/A_0) in function of time on the exponential growth phase, considering A and A_0 as optical density (OD) at time t and initial time of cultivation, respectively [30]. The acetic acid (P) yield was calculated by $Y_{P/S} = \Delta_P/\Delta_S$ (g g^{-1}) and the volumetric productivity in acetic acid was $Q_P = \Delta_{P/}\Delta t$ (g L^{-1} h^1), in which $\Delta P = P_o - P_f$ (P_o = initial acetic acid concentration and P_f = final acetic acid concentration).

3 Results and discussion

3.1 Crude glycerol treatment with different inorganic acids

The crude glycerol was submitted to treatment with concentrated inorganic acids so as to eliminate residual methanol, pigments, soaps, as well as free fatty acids. The presence of unsaturated free fatty acids and esters has an inhibitory effect on glycerol diffusion in the membrane cell, thus affecting microbial growth and bioproduct formation [18]. Table 1 shows the total volume of inorganic acid that was added to crude glycerol and its final concentration.

The neutralization of crude glycerol allowed achieving glycerol concentration of 6.2 and 5.8 % higher than glycerol grade G_8 by using phosphoric acid (H_3PO_4) and hydrochloric acid (HCl), respectively. By decreasing pH to 4.0 (G_4), it was observed that, in all studied treatments, its final concentration was higher than 900 g L^{-1}, with no significant difference from the treatment using H_2SO_4 or HCl (Table 1). The utilization of H_3PO_4 has shown to be at least 6 % more effective than the other acids at removing impurities, due to achieving a final glycerol concentration of 964 ± 3 g L^{-1}. Further addition of acid (G_2) has not shown any considerable increase in final glycerol concentration (962 ± 4 g L^{-1}) (no statistical difference between G_4 and G_2, $p > 0.05$) (Table 1). This result is similar to those observed at pH 4.0 when the glycerol generated by biodiesel from animal fat was treated with concentrated phosphoric acid [23]. Hájek and Skopal [31] achieved a glycerol concentration which is close to those observed in this study (≈86 %, *w*/*w*) by using the same concentrated inorganic acids. Table 1 also shows the amount of acids that were used to decrease pH values during the treatments. At a pH range of 2 to 4 units, more acid was used to decrease its value, though no significant improvement was observed in the pretreated glycerol concentration for each acid in particular.

3.2 Glycerol characterization

Glycerol samples treated with three inorganic acids at pH 4 (G_4) was used for further studies because it presented the highest glycerol concentration. Table 2 shows the concentration of various elements detected in crude and processed glycerol treated with inorganic acids.

Glycerol grades G_8 and G_4 contain a considerable amount of potassium, magnesium, nitrogen, and phosphorous, which can be used as nutrients for *Lactobacillus* strain growth. As expected, the glycerol treated with phosphoric acid contains a phosphorous concentration which is 11.5 times higher (397 mg L^{-1}) than that with crude glycerol (34.3 mg L^{-1}). Besides the fact that phosphoric acid removes soap, it also contributes to providing a considerable amount of phosphorus for microbial metabolism. Furthermore, the concentration of essential nutrients such as iron (42.2–53.8 mg L^{-1}), copper (1.10–1.88 mg L^{-1}), and nickel (0.40–0.47 mg L^{-1}) was quantified. These elements play an important role as co-factors in metabolic pathways and regulatory systems of bacterial cells [32].

Table 1 Glycerol concentration after treatment with inorganic acids

		Treatment					
		H_3P0_4 (85 %)		HCl (37 %)		H_2S0_4 (95 %)	
Sample	Final pH	Glycerol[a] (g L^{-1})	Volume[b] % (*v* v^{-1})	Glycerol[a] (g L^{-1})	Volume[b] % (*v* v^{-1})	Glycerol[a] (g L^{-1})	Volume[b] % (*v* v^{-1})
G_8	8.0	706 ± 1a	0	706 ± 4a	0	706 ± 5a	0
G_7	7.0	750 ± 5a	1.4	747 ± 7a	1.2	715 ± 1b	0.8
G_6	6.0	830 ± 2a	3.0	815 ± 9b	2.6	831 ± 4a	1.1
G_5	5.0	894 ± 6a	4.2	860 ± 2b	3.8	879 ± 3c	1.5
G_4	4.0	964 ± 3a	6.2	908 ± 2b	5.0	900 ± 9b	1.8
G_2	2.0	962 ± 4a	10.6	910 ± 2b	9.8	901 ± 6b	2.4

Values are means of duplicate experiments. Values with the same letter in rows showed no significant difference at 95 % confidence level

G_{suffix} glycerol at different pH (8.0; 7.0, 6.0, 5.0, 4.0, 2.0), G_8 crude glycerol (pH = 8.0)

[a] Final glycerol concentration

[b] Volume of concentrated acids (%) utilized for each 250 mL of crude glycerol

Table 2 Concentrations of elements in glycerol samples

Sample	Element (mg L^{-1})[a]												
	Ni	Cu	Fe	Cd	As	Pb	Al	Ca	Mg	Na	K	N	P
Crude glycerol	0.48 ± 0.03	1.88 ± 0.02	53.8 ± 0.1	0.05 ± 0.02	0.10 ± 0.03	BDL	243 ± 0.5	27.3 ± 0.01	64.5 ± 0.8	0.05	187 ± 0.2	49.6 ± 0.2	34.3 ± 0.1
G_4 H_3PO_4 (85 %)	0.40 ± 0.02	1.10 ± 0.01	50.8 ± 0.2	BDL	BDL	BDL	16.8 ± 0.2	3.03 ± 0.01	33.3 ± 0.2	BDL	150 ± 0.1	43.9 ± 0.1	397 ± 0.1
G_4 HCl (37 %)	0.41 ± 0.01	1.08 ± 0.01	42.2 ± 0.3	BDL	BDL	BDL	73.2 ± 0.4	2.86 ± 0.02	65.3 ± 0.2	BDL	178 ± 0.1	44.5 ± 0.4	8.7 ± 0.2
G_4 H_2SO_4 (98 %)	0.47 ± 0.02	1.46 ± 0.01	48.4 ± 0.1	BDL	BDL	BDL	80.2 ± 0.5	1.85 ± 0.01	7.8 ± 0.1	BDL	120 ± 0.1	50.1 ± 0.3	28.1 ± 0.2

G_4 treated glycerol (final pH 4.0), *BLD* below detection limit (<0,05 mg L^{-1})

[a] Values are means of duplicate analysis

Heavy metals such as arsenic, cadmium, and lead derived from potential metal loss during the biodiesel production process were not detected in the samples. These elements and its ions could exert a negative impact on the growth and viability of microorganisms, which could negatively affect the utilization of glycerol as substrate for medium formulation or food formulation [33]. In addition, sodium was not detected in all analyzed samples, once potassium hydroxide (KOH) was used as catalyst in the biodiesel production process.

Removal of soap and free fatty acid from glycerol G_4 was confirmed by Fourier-transformed infrared (FTIR) analysis. FTIR spectroscopy has been reported as an accurate method to identify functional groups by detecting covalent links in high-density liquid samples, such as soybean oil and esters [34]. Figure 1 shows the absorption spectra of crude glycerol and pure substances. The broad absorption band associated with hydroxyl groups of glycerol is at 3356 cm^{-1} and carbon-oxygen absorptions, which characterize primary and secondary alcohols, were observed at 1044 and 1112 cm^{-1}, respectively.

The peak around 1744 cm^{-1} wavenumber in pure soybean oil spectra (Fig. 1f) represents the carbonyl group (C = 0), which is characteristic of fatty acid spectra [27, 35]. The peak observed in the range of 1550 cm^{-1} corresponds to an ionized form of pure potassium oleate and linoleate spectra (Fig. 1c–e), which are the most abundant fatty acids in soybean oil [28, 36]. Similar functional groups observed in samples of crude glycerol show the presence of free fatty acids and soap. The treated glycerol spectrum shows the treatment effectiveness at removing soap with concentrated inorganic acids, which could be concluded due to the absence of ionized ester in the spectrum (Fig. 1b). The presence of free fatty acids, methyl esters and soaps negatively affect the permeability of cell membrane, morphology, and cellular development of many microorganism species, including yeast and bacteria [14, 16–18]. These results showed that the treatment of crude glycerol with phosphoric acid at pH 4 (G4) is effective at removing impurities.

3.3 Screening of probiotic *Lactobacillus* strains for glycerol assimilation

Fifteen potential probiotic *Lactobacillus* strains that had been previously identified by Sumita [37] were used to assess their ability to metabolize glycerol as main carbon and energy source. Firstly, it was used a modified MRS medium containing crude glycerol (in the range of 5 to 25 g L^{-1}), where the absence of cellular growth was observed in all tested lactobacilli strains, with the exception of *L. delbrueckii* UFV-H2b20 that provided slight biomass growth (optical density OD_{600nm} was lower than 0.06, data not shown). A different profile was verified by cultivating the lactobacilli strains on modified MRS medium with treated glycerol instead of crude glycerol, as shown by the growth parameters and

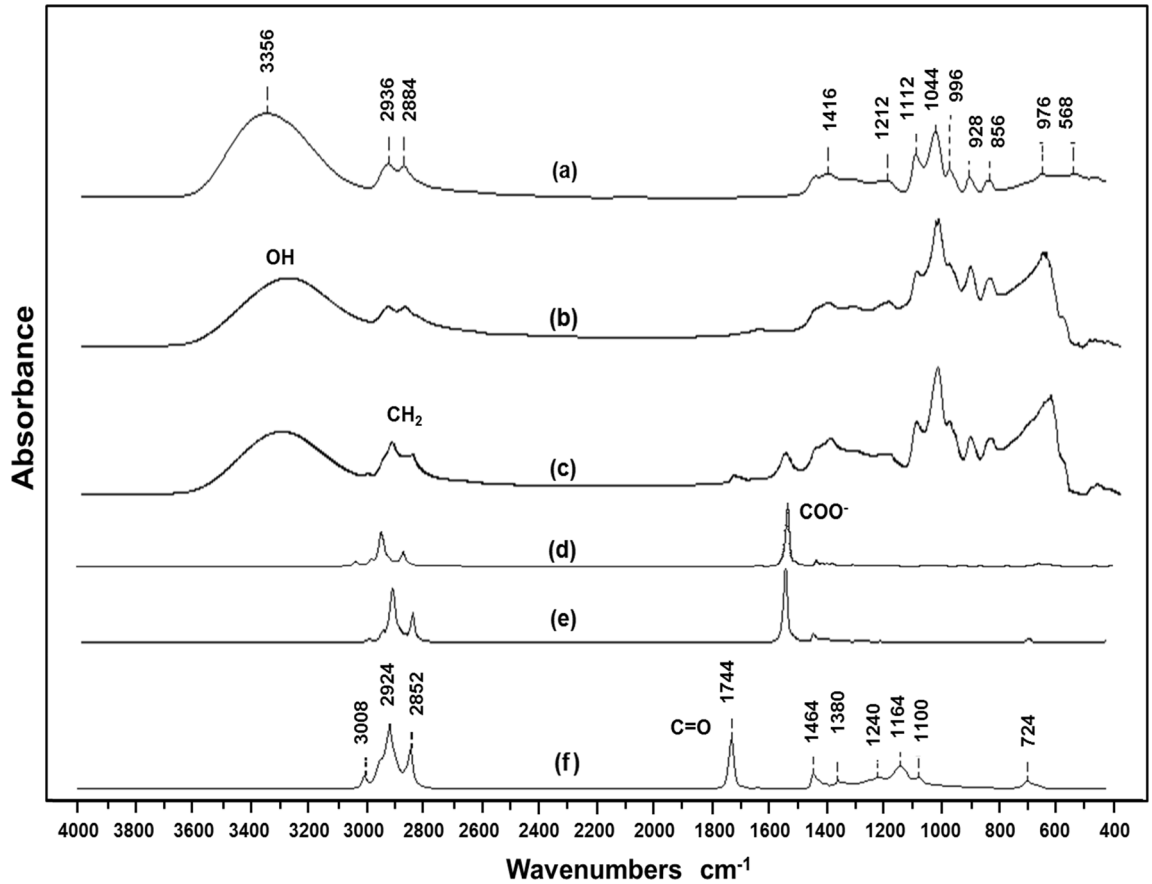

Fig. 1 FTIR spectra (4000 to 400 cm^{-1}) of pure glycerol (**a**); treated glycerol-G_4 (**b**); crude glycerol (**c**); potassium linoleate (**d**); potassium oleate (**e**), and soybean oil (**f**)

glycerol consumption. Table 3 displays the values of total lactobacilli viable cells and biochemical parameters, such as $Y_{X/S}$ and Q_X, after 24 h of cultivation. Maximal biomass production was used as selection criteria for further experiments.

It can be noted that almost all tested strains had the ability to grow in a medium containing treated glycerol, except for *L. paracasei*. It should also be observed that the highest cell concentration was achieved by *L. delbrueckii* UFV-H2b20 (0.82 g L^{-1}), followed by *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 with 0.73 g L^{-1} and 0.72 g L^{-1}, respectively (Table 3). Lactic acid bacteria, such as several strains of *Lactobacillus*, *Pediococcus*, *and Enterococcus*, can metabolize glycerol under aerobic and anaerobic conditions [9, 19–22]. Alvarez et al. [19], reported similar biomass concentration for *Lactobacillus rhamnosus* being grown in 3.9 g L^{-1} glycerol under microaerobic conditions. These authors also confirmed the ability of *Lactobacillus casei* ATCC 393, *Lactobacillus zeae* ATCC 15820, and *L. rhamnosus* ATCC 15820 to use glycerol as sole carbon source. Pasteris and Strasser de Saad [20] reported that when *Pediococcus pentosaceus* was cultivated in a medium containing glycerol (4.0 g L^{-1}), the biomass produced (0.56 g L^{-1}) was comparable to that obtained with glucose. Garai-Ibabe et al. [38] reported that *Lactobacillus collinoides and Lactobacillus diolivorans* isolated from spoiled cider were tested for their capability to grow in a medium containing glycerol. Under microaerobic and anaerobic conditions, these species were able to degrade glycerol in the range of 12 to 90 % of its initial concentration, thus producing biomass and 1,3-propanediol. The *Lactobacillus* strain behavior studied in this work was also described in a few previously published works.

With regard to the $Y_{X/S}$ parameter, values ranging from 0.08 to 0.34 g g^{-1} were achieved (Table 3). Based on their highest biomass production in comparison with other lactobacilli strains studied, *L. delbrueckii* UFV-H2b20, *L. acidophilus* ATCC 4356 and *L. plantarum* ATCC 8014 were selected for further experiments.

3.4 Kinetic studies of selected strains

Kinetics of growth, glycerol consumption, pH, and acetic acid production of the lactobacilli grown in an unbuffered medium and in media buffered at pH 6.0 are shown in Fig. 2. During incubation in unbuffered medium containing treated glycerol, *L. delbrueckii* UFV-H2b20 provided the highest cell concentration (0.88 g L^{-1}) in comparison with *L. plantarum* ATCC

Table 3 Biochemical parameters after a 24-h growth of lactobacilli in a medium containing glycerol

Bacteria	Strain	Biomass (g L^{-1})	Cell (CFU mL^{-1})[a]	$Y_{X/S}$ (g g^{-1})	Q_X (g L^{-1} h^{-1})	Substrate consumption (%)
L. fermentum	LAC 01	0.59 ± 0.03	$1.77x10^8$	0.14	0.025	20
L. casei	LAC 04	0.38 ± 0.12	$1.47x10^8$	0.08	0.016	23
L. plantarum	LAC 06	0.40 ± 0.01	$1.56x10^8$	0.10	0.017	19
L. fermentum	LAC 07	0.56 ± 0.03	$1.64x10^8$	0.17	0.023	16
L. fermentum	LAC 09	0.45 ± 0.08	$1.82x10^8$	0.16	0.019	14
L. plantarum	LAC 19	0.47 ± 0.04	$1.71x10^8$	0.15	0.019	15
L. plantarum	LAC 23	0.33 ± 0.11	$1.24x10^8$	0.10	0.014	17
L. plantarum	LAC 30	0.40 ± 0.07	$1.55x10^8$	0.17	0.017	11
L. fermentum	LAC 38	0.41 ± 0.02	$1.63x10^8$	0.18	0.017	11
L. plantarum	LAC 40	0.54 ± 0.01	$2.05x10^8$	0.12	0.023	22
L. paracasei	LAC PC1	–	–	–	–	0
L. paracasei	LAC PC2	–	–	–	–	0
L. delbrueckii	UFV-H2b20	0.82 ± 0.02	$2.82x10^8$	0.34	0.034	12
L. acidophilus	ATCC 4356	0.73 ± 0.04	$2.43x10^8$	0.28	0.031	13
L. plantarum	ATCC 8014	0.72 ± 0.01	$2.35x10^8$	0.26	0.030	13

Cultivation carried out in modified MRS medium containing treated glycerol with H_3PO_4 (20 g L^{-1}) at 37 °C for 24 h

$Y_{X/S}$ biomass yield, Q_X biomass productivity

[a] Values obtained from pour plate method at 37 °C for 72 h. Values are means of triplicate experiments

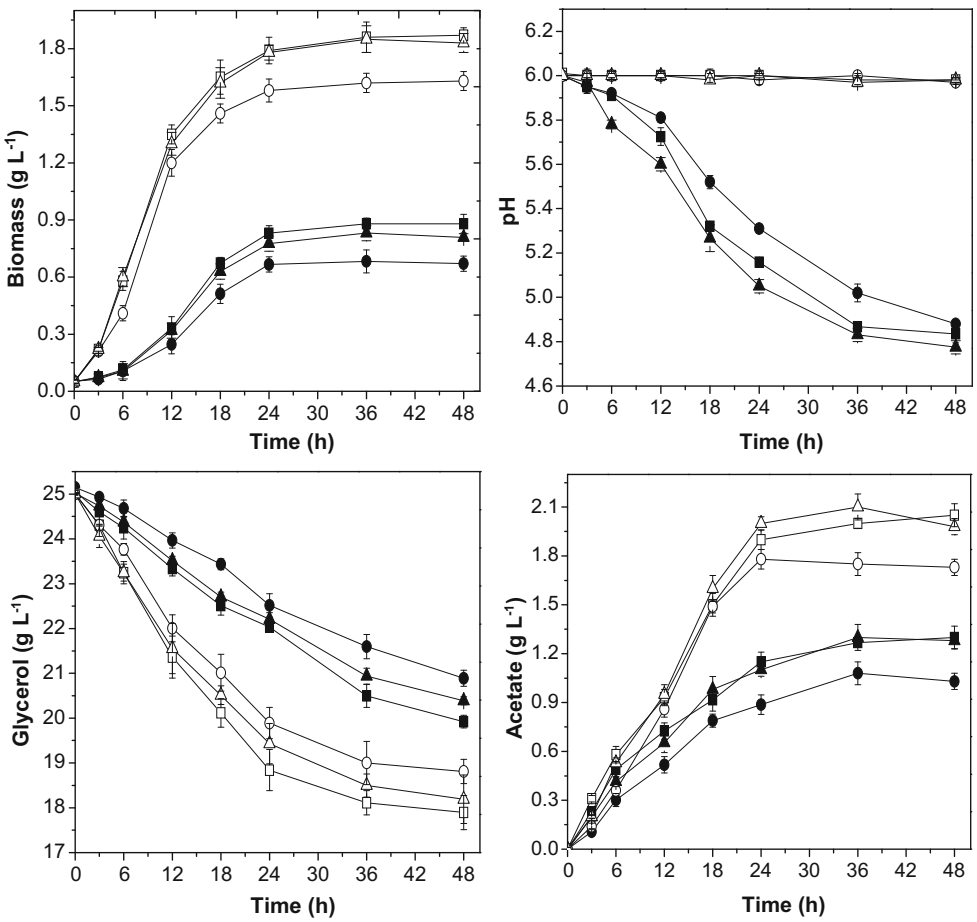

Fig. 2 Kinetics of growth and acetic acid production for *L. delbrueckii* UFV-H2b20 (*square symbol*), *L. acidophilus* ATCC 4356 (*triangle symbol*) and *L. plantarum* ATCC 8014 (*circle symbol*) in medium containing glycerol (25 g L^{-1}). *Closed symbol*, unbuffered medium; *opened symbol*, medium at pH 6.0

8014 and *L. acidophilus* ATCC 4356, with 0.65 g L^{-1} and 0.78 g L^{-1}, respectively. It is important to note that the growth rate of the lactobacilli in the unbuffered medium decreased when pH dropped from pH 6.0 to pH 5.0, thus affecting microorganism growth and cell viability. It is known that external pH determines the cytoplasmatic pH, thus affecting growth, enzyme activity, and stability of many molecules in bacterial cells [39]. The low glycerol assimilation and growth provided by the strains studied herein could be related to the fact that the lactobacilli species are characterized as neutrophile organisms with optimal pH for growth in the range of 6.0 to 6.5, except for *L. delbrueckii* that is able to grow at pH 5.5 [40]. Moreover, optimal pH for enzyme activities which are involved in glycerol assimilation by many microorganisms, such as glycerol kinase and glycerol dehydrogenase, ranges from 5.5–7.0 or higher [2, 19, 41]. Pasteris and Strasser de Saad [20] showed that the growth of *P. pentosaceus* was ceased after 24 h of cultivation, regardless of glycerol consumption. Such limitation was attributed to a low pH which results from acetic acid production. These results are similar to those reported by Alvarez et al. [19] during the cultivation of *L. rhamnosus* ATTC 7469 in a medium containing pure glycerol as carbon source under aerobic conditions. According to these authors, 0.46 g L^{-1} of cells were obtained, resulting in a yield of 0.19 g g^{-1} and substrate consumption of 3.92 g L^{-1}, which corresponds to 60 % of the initial carbon source. The authors also observed a biphasic growth of *L. rhamnosus* during the co-fermentation of glycerol and glucose. In this condition, glycerol consumption was initiated after glucose exhaustion, thus achieving 0.58 g L^{-1} biomass. Similar cell density was observed in genetically transformed *Lactobacillus panis* containing an artificial glycerol oxidative pathway, cultured in modified MRS medium and supplemented with 24 mM glycerol (2.2 g L^{-1}). The engineered strain was able to metabolize almost 50 % of the initial glycerol concentration after 24 h of cultivation, thus resulting in a final cell density (OD_{600nm}) of 0.5 [42].

The buffered medium promoted an increase in the performance of all strains analyzed, due to attaining cell concentration in the range of 1.55 g L^{-1} and 1.74 g L^{-1}. The highest specific growth rate (μ, h^{-1}) values were in the range of 0.23, 0,24, and 0.25 h^{-1} for *L. delbruecki* UFV-H2b20, *L. plantarum* ATCC 8014, and *L. acidophilus* ATCC 4356, respectively (Table 4). These values are 50 % higher than those observed in an unbuffered medium (Table 4). The cell volumetric productivity (Q_X) was similar for all studied strains during 24 h of cultivation. Regarding substrate concentration, glycerol was not consumed completely by all studied bacteria, whose consumption was in a range of 10 to 12 % and 21 to 24 % of the glycerol that had been initially supplied to the unbuffered and buffered media, respectively (Table 4). Cultures conducted with pure glycerol (25 g L^{-1}) at same conditions showed slightly lower values of cell concentration (1.45–1.68 g L^{-1}) and glycerol utilization (18–21 %) in comparison with that observed with the buffered medium containing treated glycerol (data not shown).

Furthermore, all strains assessed herein showed the ability to produce acetate at a concentration of up to 2.00 g L^{-1} in the buffered medium. The acetate synthesis in *Lactobacillus*

Table 4 Biochemical parameters of lactobacilli growing in glycerol medium

Biochemical parameter	*L. delbrueckii* UFV-H2b20		*L. plantarum* ATCC 8014		*L. acidophilus* ATCC 4356	
	A	B	A	B	A	B
Biomass, g L^{-1}	0.88 ± 0.02	1.76 ± 0.07	0.65 ± 0.03	1.55 ± 0.08	0.78 ± 0.01	1.74 ± 0.04
Glycerol consumption, g L^{-1}	3.10 ± 0.03	5.70 ± 0.40	2.60 ± 0.05	5.30 ± 0.29	2.85 ± 0.12	6.00 ± 0.25
$Y_{X/S}$, g g^{-1}	0.29 ± 0.01	0.31 ± 0.01	0.25 ± 0.02	0.29 ± 001	0.28 ± 0.01	0.29 ± 0.01
Q_X, g L^{-1} h^{-1}	0.03 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.07 ± 0.02
Q_S, g L^{-1} h^{-1}	0.12 ± 0.02	0.23 ± 0.02	0.11 ± 0.02	0.22 ± 0.01	0.12 ± 0.01	0.25 ± 0.01
μ_{max}, h^{-1}, (r^2)	0.13 (0.96)	0.24 (0.94)	0.12 (0.96)	0.23 (0.96)	0.13 (0.97)	0.24 (0.92)
g_t, h	5.33 ± 0.05	2.88 ± 0.01	5.76 ± 0.07	2.77 ± 0.01	5.33 ± 0.03	2.77 ± 0.02
Acetate, g L^{-1}	1.29 ± 0.05	1.90 ± 0.02	0.88 ± 0.03	1.78 ± 0.06	1.25 ± 0.02	2.00 ± 0.04
$Y_{P/S}$, g g^{-1}	0.41 ± 0.03	0.33 ± 0.02	0.34 ± 0.02	0.34 ± 0.01	0.43 ± 0.02	0.33 ± 0.02
Q_P, g L^{-1} h^{-1}	0.05 ± 0.02	0.08 ± 0.02	0.04 ± 0.02	0.07 ± 0.01	0.04 ± 0.02	0.08 ± 0.01

Cultivation carried out in modified MRS medium containing glycerol treated with H_3PO_4 (25 g L^{-1}) at 37 °C for 24 hours

A unbuffered medium, *B* pH 6.0 (potassium phosphate buffer,0.2 M), $Y_{X/S}$ cell mass yield (where *X* is the cell mass produced and *S* is the substrate consumed), Q_X volumetric biomass productivity, Q_S volumetric glycerol consumption, μ_{max} maximum specific growth rate, r^2 linear regression coefficient, g_t generation time (ln2/μ), $Y_{P/S}$ acetic acid yield, Q_P acetic acid productivity

strains could be associated with the activation of pyruvate dehydrogenase complex (PDH) under microaerobic condition and acetate kinase (ACK) pathways [43]. Acetic acid yield values ranged from 0.33 to 0.43 g g^{-1} and the volumetric productivity (Q_P) was similar after 24 hours of cultivation in the unbuffered and buffered medium (Table 4).

In a previous work, acetic acid production (≈1.78 g L^{-1}) was reported with the recombinant bacteria *L. panis*, which was cultured in modified MRS medium containing 160 mM glycerol (14.7 g L^{-1}) for 24 hours under uncontrolled pH conditions [42]. Alvarez et al. [19], while culturing the probiotic strain *L. rhamnosus* in pure glycerol, reported acetate concentration (0.66 g L^{-1}) which is 25 % lower than those observed in the present work (unbuffered medium), yielding 0.16 g g^{-1} acetic acid. The authors also reported the production of 1.27 g L^{-1} of acetic acid during the co-fermentation of glycerol and glucose by *L. rhamnosus*. Similar results were found through the present work by using glycerol as the main substrate. Pasteris and Strasser de Saad [21] have produced 0.24 g L^{-1} of acetic acid with 12.5 % glycerol consumption by *L. hilgardii* in the co-fermentation of glycerol and glucose. When glycerol was used as carbon source at different concentrations, *L. pentosaceus* produced acetic acid at a concentration of 0.86 g L^{-1} [20]. Nonetheless, the amount of biomass and acetic acid produced in the present work is still lower in comparison with those produced by some yeast strains in glycerol. For example, the yeast *Yarrowia lipolytica* while it was being grown with glycerol (30 g L^{-1}) under nitrogen-limited conditions, secreted 9.2 g L^{-1} acetic acid and produced 7.1 g L^{-1} of biomass [24].

Pflügl et al. [25] reported the production of acetic acid in a range of 5.3 to 19.5 g L^{-1} by co-fermenting glycerol (10 to 70 g L^{-1}) and glucose (30 g L^{-1}). Such results demonstrate that glycerol-sugar co-fermentation enhances the assimilation rate of glycerol and acetic acid production. In a more recent work, these authors conducted cultures of *L. diolivorans* in a bioreactor by using neutralized raw glycerol (10 g L^{-1}) and glucose (20 g L^{-1}) as substrates, thus achieving 6.7 g L^{-1} concentration of acetic acid [26]. In a glycerol-glucose co-fermentation by *L. reuteri* DSM 20016 for 1,3-propanediol production, 2.9 g L^{-1} biomass and 12.9 g L^{-1} acetic acid was reported [44]. According to literature, co-fermentation could be a strategy to increase glycerol assimilation by the strains studied herein.

In this work, it was demonstrated that *L. delbruecki* UFV-H2b20, *L. plantarum* ATCC 8014, and *L. acidophilus* ATCC 4356 are able to grow with glycerol being used as sole substrate.

4 Conclusion

Results of glycerol treatments have revealed that all analyzed inorganic acids allowed obtaining a glycerol concentration that was higher than 900 g L^{-1} after decantation. Besides soap removal from crude glycerol, phosphoric acid treatment provides phosphorous in the culture medium for cell growth. *L. delbrueckii* UFV-H2b20, *L. plantarum* ATCC 8014, and *L. acidophilus* ATCC 4356 yielded the highest biomass production. Regarding the kinetic analysis, the batch culture data of *L. delbrueckii* UFV-H2b20 and *L. acidophilus* ATCC 4356 showed a similar performance for biomass and acetic acid production in media containing glycerol and phosphate buffer. These results will contribute to promoting the potential use of glycerol derived from biodiesel production as substrate for lactobacilli growth.

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