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# Bioconversion of Glycerol into Lactic Acid by a New Bacterial Strain from the Brazilian Cerrado Soil

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**Abstract:** A lactic-acid-producing strain was isolated from the Brazilian Cerrado soil (Brazilian savanna). Glycerol, a byproduct of the biodiesel industry, can be converted into various chemical intermediates of industrial value by biotechnological routes. *Klebsiella pneumoniae* can metabolize glycerol in environments with or without oxygen and bioconvert it into several chemicals with high value-added, such as lactic acid, 3-hydroxypropionic acid and 1,3 propanediol. The wild-type bacterial strain (2GPP) isolated from a soil sample from the Brazilian Cerrado was determined to be a *K. pneumoniae* complex that was capable of successfully metabolizing glycerol. Fermentations were performed with different temperatures, pH, and inoculum concentrations to evaluate the best lactic acid production. At first, 1,3-propanediol and L-(+)-lactic acid were produced in mini reactors. A lactic acid production of 3.8 $g \cdot L^{-1}$ and a decrease in 1,3-propanediol output were observed. Thus, by adjusting process variables such as pH and temperature during fermentation, it was possible to maximize the production of lactic acid and decrease the formation of 1,3-propanediol by utilizing experimental design strategies.

**Keywords:** lactic acid; glycerol; *Klebsiella pneumoniae*; fermentation; brazilian cerrado soil



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## 1. Introduction

The production of fuels from renewable raw materials is an essential strategy to meet the global energy demand, providing a potential alternative to fossil fuels [1,2]. Biodiesel is an example of a biofuel. A byproduct, glycerol, is formed during its production. The disposal of this byproduct causes damage to the environment, so there is considerable interest in developing studies looking at how to add value to glycerol [3–5]. Thus, there is an interest in the bio-conversion of the glycerol produced in higher-value products, improving the process economy and minimizing waste [6,7]. Glycerol is already a raw material source of many high-value products such as polymers, fuel additives, antibiotics, and painkillers. Microorganisms can bioconvert glycerol into metabolites due to the size and structure of their molecules, with similar yields to those obtained with the sugar metabolism [3]. The glycerol molecules can cross the cytoplasmic membrane by passive diffusion.

Fermentative glycerol metabolism has been studied in detail in bacteria, including *Citrobacter*, *Klebsiella*, *Clostridium*, *Lactobacillus*, and even *Shimwellia*, to obtain high-value products, such as 1,3-propanediol, 2,3-butanediol, ethyl alcohol and hydrogen. In *Klebsiella*, glycerol can be metabolized both oxidatively and reductively. *K. pneumoniae*, a facultative anaerobe bacterium, is capable of bioconverting glycerol molecules in both aerobic and anaerobic environments. The reductive anaerobic metabolism of glycerol produces 1,3-propanediol, while the oxidative anaerobic metabolism of glycerol produces pyruvate and

contributes to cell (biomass cellular) growth. The end products of pyruvate-deriving are lactic acid, ethanol, and 2,3-butanediol [3,4,6,8–11].

*K. pneumoniae*, with its broad substrate characteristics, high yield, and easy cultivation, has been an efficient type of bacteria for 2,3-butanediol production [12]. However, there are also studies showing that such a microorganism could metabolize substances such as 1,3-propanediol, 2-ketogluconic acid, succinate, 3-hydroxypropionic acid and ethanol [12–18]. Figure 1 shows some possible end products of glycerol degradation.

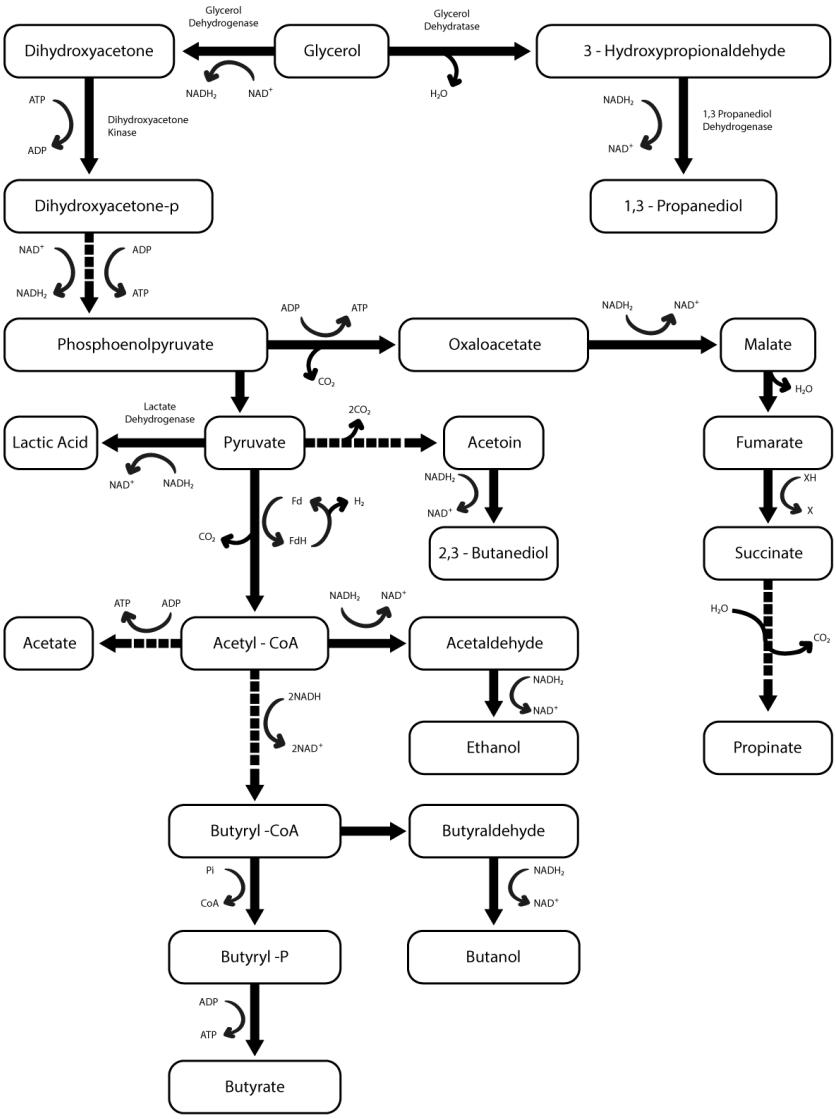


**Figure 1.** Schematic pathways of glycerol metabolism from some microorganisms. Adapted with permission from da Silva et al. [19]. Copyright 2008 © Elsevier Inc.

Lactic acid (LA), an essential monomer in the polymer industry, can be obtained by bioconversion through microbial fermentation (bacteria, fungi, and yeast) and chemical synthesis. In the chemical route, the isomers D-(−)-lactic acid and L-(+)-lactic acid are produced. The main advantages of this method are the lower cost of the purification step and the absence of a residual substrate. The advantages of fermentation are the possibility of using low-cost raw materials to produce one specific stereospecific enantiomer of the isomers' lactic acid by controlling the process parameters. The L-lactic acid enantiomeric form is the most common for applications in the polymer industry. As a matter of fact, high optical and chemical purities are considered important requirements for commercial lactic acid applications [4,10,20–23].

This compound has diverse applications, including as a pH adjusting agent, active ingredient in cosmetics, and monomer in biodegradable polymer poly(lactic acid) production. Lactic acid polymers (PLA) can also be used in medicine due to their resorption capacity. Therefore, they are used as pins and plates for bone implants, sutures and tissue regeneration [4,17,19].

PLA is a thermoplastic biopolymer that can be semicrystalline or amorphous in nature. In this scenario, the bioconversion of renewable resources to produce lactic acid exhibiting high optical purity is fundamental to improving the physical characteristics of PLA, such as crystallization, thermostability, and mechanical performance. The mechanisms PLA biodegradation involve a two-step degradation process. First, the compost's heat and moisture attack the PLA chains, splitting them apart until lactic acid is produced. After that, the microorganisms in the soil mineralize the polymer fragments and lactic acid into methane under anaerobic conditions and carbon dioxide under aerobic conditions [24–26]. Table 1 shows examples of microorganisms capable of metabolizing glycerol to lactic acid.

**Table 1.** Examples of fermentation with glycerol as a carbon source to obtain lactic acid.

| Microorganism | Substrate | Lactic Acid Concentration ($g \cdot L^{-1}$) | Yield ($g \cdot g^{-1}$) | Productivity ($g \cdot L^{-1} \cdot h^{-1}$) | Reference |
|---|---|---|---|---|---|
| *Komagataella phiffi Strains* | glycerol | 70.0 | 0.665 | 1.39 | [27] |
| *Escherichia coli AC-521* | glycerol | 90.4 | 0.88 | 1.13 | [28] |
| *Enterococcus faecalis* | waste glycerol from biodiesel production | 15.8 | 0.37 | 0.61 | [29] |
| *Lactobacillus reuteri DSM 20,016 Δadh2* | glycerol and glucose | 50.44 | 0.78 | 1.50 * | [30] |
| *Enterococcus faecalis strain W11* | glycerol | 135.0 | 0.9 | 0.87 | [31] |
| *Lactobacillus reuteri* | glycerol and glucose | 6.0 | 0.536 | 0.28 | [32] |

* $mM \cdot mL^{-1} \cdot h^{-1}$.

To decrease the cost of the raw materials needed for fermentation, several strategies are being investigated. Different wastes have also been used to produce lactic acid, for instance, for the production of L-(+)-lactic acid with high enantiomeric purity, achieved by using *B. coagulans* A166, López-Gómez et al. [22] have analyzed the possibility of fermentation with hydrolysates derived from the organic fraction of a municipal solid waste, resulting in 61.1 $g \cdot L^{-1}$ of lactic acid and a yield of 0.94 $g \cdot g^{-1}$. Ma et al. [23] used an agricultural lignocellulosic biomass (rice straw) as a fermentation substrate to produce D-(−)-lactic acid by employing *Lactobacillus delbrueckii*, producing 46.6 $g \cdot L^{-1}$ of lactic acid, and a yield of 0.92 $g \cdot g^{-1}$ with 99.5% of optical purity. Bai et al. [33] also evaluated the production of D-(−)-lactic acid by *Sporolactobacillus inulinus* YBS1–5, using cottonseed meal and corncob residue, reaching 107.2 $g \cdot L^{-1}$ of lactic acid with a yield of 0.85 $g \cdot g^{-1}$.

Lactic acid is also an interesting alternative to acrylic acid and alkyl acrylate production from renewable sources. It is possible to produce acrylates by dehydrating lactic acid, using heterogeneous catalysis with zeolites and hydroxyapatite, minimizing the dependence on petrochemical sources to produce important acrylate-based monomers [34–36].

In this context, this research aimed to isolate new strains of bacteria capable of bioconverting glycerol into lactic acid and 1,3-propanediol, molecules of high biotechnological interest. The present work was able to isolate and identify a wild bacteria strain from the Brazilian biome (*K. pneumoniae*), which was capable of metabolizing glycerol to L-(+)-lactic acid with 100% optical purity and 1,3-propanediol, without requiring genetic modification of the microorganism. The study directed the main metabolic pathway of the microorganism to produce the product of interest (lactic acid) by changing fermentation parameters such as pH, temperature and inoculum concentration. In addition, a factorial design strategy was used to verify the optimal conditions for glycerol consumption and L-(+)-lactic acid production. Although 1,3-propanediol is a value-added chemical with numerous applications in the food, cosmetics and textile sectors, in the present work, we chose to control and optimize the generation of lactic acid. The production of just one of

the organic acids would facilitate the purification process and avoid competition for the carbon source.

## 2. Materials and Methods

### *2.1. Soil Sampling and Microbial Isolation*

For bacterial isolation, 50 mL of standard glycerol (Vetec, Rio de Janeiro, RJ, Brazil) and crude glycerol (kindly provided by Cesbra Química S/A, Volta Redonda, RJ, Brazil) (65% purity) were spilled onto the conserved (native) Cerrado soil in two different sampling points at the Embrapa Cenargen Experimental Station Fazenda Sucupira (Brasília, Federal District, Brazil): *Amaranthus* ssp. point (S: 15°54′46.3″ W: 048°02′2.1″) and dam point (S: 15°55′03 0.80″ W: 048°01′19.7″). In each location, the spill point of each glycerin was separated by approximately 1 m. Samples of the glycerin-enriched soils (collected from the uppermost layer; 0–10 cm deep) were collected after two months, placed in a sterile bag, and stored at 25 °C until bacterial isolation.

The screening of microorganisms capable of metabolizing commercial and crude glycerol was performed using tryptic soy agar (TSA, KASVI) culture medium, containing 4% each of glycerol and Benomyl fungicide solution (50 μg·mL$^{-1}$) to inhibit the fungal contaminants. The bacterial isolation was performed through sequential dilution and plating steps on the agar medium. The isolates were purified by successive restreak, and pure cultures were maintained at −80 °C in glycerol (20%), used as a cryoprotectant agent.

Eighteen bacterial strains isolated from soil enrichment with pure and crude glycerin were analyzed to determine the secreted metabolites, focusing on lactic acid. All the bacterial strains were submitted to fermentative tests, and only the one with the highest efficiency in the production of lactic acid was identified and reported in the present work.

### *2.2. Culture Conditions and Selection of Bacterial Strains Producing Lactic Acid under Microaerobiosis*

For bacterial growth, the tryptic soy agar (TSA, KASVI) medium was used. M9 broth Minimal Salts, 5X (Sigma-Aldrich Brasil Ltda, Barueri, SP, Brazil) with glycerol (99.5%, Sigma-Aldrich Brasil Ltda, Barueri, SP, Brazil) was used in fermentations. From pure colonies isolated on Petri dishes, the pre-culture inoculum was performed using 25 mL of M9 broth supplemented with 4% glycerol as a carbon source, added in 125 mL conical flasks to each bacterial strain at 120 rpm and 30 ± 1 °C for 24 h.

Then, 800 μL of culture medium were dispersed in each well of three 96-well microplates of 1.1 mL in volume, and 200 μL of each bacterium was inoculated in separate wells of each plate, in duplicate. The microplates were maintained in a rotatory shaker at 28 °C, 200 rpm, for 24 h. Then, the broths were centrifuged for cell separation at 14,000 rpm for 10 min (Eppendorf, 5418 R, Eppendorf do Brasil Ltda., São Paulo, SP, Brazil). The supernatants were separated to quantify value-added biocomposites by the high-performance liquid chromatography (HPLC) method (Agilent, 1260 infinite). This last step was carried out in every bioconversion step. The 2GPP strain was selected for subsequent experiments.

### *2.3. 16S rRNA Gene Analysis*

The selected strain, 2GPP, was cultivated in 10 mL of tryptic soy broth (TSB, KASVI) at 30 °C and 150 rpm for 24 h. Cells were harvested through centrifugation, and the genomic DNA was purified using Wizard® Genomic DNA Purification Kit (Promega). A partial sequence of the 16S rRNA gene was PCR amplified with the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [37,38]. The purified products were sequenced in both directions using ABI BigDye terminator chemistry at the Eurofins company (Indaiatuba, São Paulo, Brazil).

The consensus sequence was generated using Geneious R11 (https://www.geneious.com, Brasília, DF, Brazil, 31 October 2021), and the resulting sequence was aligned with 30 representative sequences of different *Klebsiella* species (type strains) retrieved from GenBank (NCBI database). The sequences were aligned using a multiple sequence alignment

computer program, MAFFT, as described by Katoh et al. [39]. A maximum likelihood (ML) phylogenetic tree was obtained by using a phylogeny computer program, PhyML 3.0, developed by Guindon et al. [40], and the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT) was employed to estimate the node support [41].

### *2.4. Evaluation of Inoculum Concentration for the Lactic Acid Synthesis*

A new experiment was carried out with the 2GPP strain. A total of 10 mL of M9 broth medium supplemented with 4% (mass fraction) crude glycerol was added to 15 mL flasks and, in laminar flow, inoculated with the cream of the bacteria at concentrations of 5.0, 4.2, 2.0, and 1.0 $g \cdot L^{-1}$. The flasks were kept in a rotary shaker at 32 °C and 120 rpm for 48 h. The control flask tube was kept unstirred to verify the influence of such a factor on fermentation at a concentration of 4.2 $g \cdot L^{-1}$. Then, 1 mL aliquots were taken at 24 and 48 h.

### *2.5. Experimental Design*

The fermentations based on the experimental design were inoculated in 125 mL conical flasks using 100 mL of M9 broth medium, supplemented with 4% (rotatable central composite design) and 10% (full factorial design) crude glycerol. The vials containing the medium were autoclaved, and the microorganism was inoculated in the flasks and kept in a rotary shaker at 50 rpm. Statistical analysis was carried out using the TIBCO Statistica® 13.3.0. with a significance level (p) equal to 0.05. Parametric data analysis was carried out using analysis of variance (ANOVA).

#### 2.5.1. Factorial Design

The experimental design was carried out using independent variables (temperature, inoculum concentration, and pH), which can be expressive and determine the 2GPP strain's growth as lactic acid production for 48 h. Full factorial design $2^3$ with replicates at the central point was employed to evaluate and determine factors that significantly influence fermentation and lactic acid production. This experimental planning also contemplates the experimental runs carried out at the central point in triplicate, intended for the evaluation of experimental error and curvature check (quadratic contribution evaluated at the central point of the experimental planning), as shown in Table 2.

**Table 2.** $2^3$ factorial design, with three replicates at the central point and factor level codes for the fermentations, performed with the selected strain *Klebsiella* sp. 2GPP.

| **Variables** | **−1** | **0** | **+1** |
|---|---|---|---|
| $X_1$: Temperature (°C) | 27 | 32 | 37 |
| $X_2$: Inoculum concentration ($g \cdot L^{-1}$) | 5 | 12.5 | 20 |
| $X_3$: pH | 6.4 | 7.4 | 8.5 |
| Experiment | $X_1$ | $X_2$ | $X_3$ |
| 1 | −1 | −1 | −1 |
| 2 | +1 | −1 | −1 |
| 3 | −1 | +1 | −1 |
| 4 | +1 | +1 | −1 |
| 5 | −1 | −1 | +1 |
| 6 | +1 | −1 | +1 |
| 7 | −1 | +1 | +1 |
| 8 | +1 | +1 | +1 |
| 9 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 |

#### 2.5.2. Rotatable Central Composite Design

Fermentations were performed in 125 mL conical flasks using M9 broth, supplemented with 4% glycerol as a culture medium. This planning also contemplates the use of a triplicate

at the central point to determine the experimental error. The pH and the concentration of the inoculum were performed, as shown in Table 3. The experiment was carried out at 37 °C for 72 h. The pH was initially corrected by adding 0.1 M NaOH solution (according to Table 3), and fermentations were carried out without pH control.

**Table 3.** Conditions of the rotatable central composite design (RCCD) experiments and factor level codes.

| **Variables** | **−1.41** | **−1** | **0** | **+1** | **+1.41** |
|---|---|---|---|---|---|
| X1: Inoculum concentration ($g \cdot L^{-1}$) | 6.1 | 7.5 | 10 | 12.5 | 14.9 |
| X2: pH | 7.6 | 9 | 9.5 | 10 | 11.4 |
| Experiment | | $X_1$ | | $X_2$ | |
| 1 | | −1 | | −1 | |
| 2 | | +1 | | −1 | |
| 3 | | −1 | | +1 | |
| 4 | | +1 | | +1 | |
| 5 | | 0 | | 0 | |
| 6 | | 0 | | 0 | |
| 7 | | 0 | | 0 | |
| 8 | | −1.41 | | 0 | |
| 9 | | +1.41 | | 0 | |
| 10 | | 0 | | −1.41 | |
| 11 | | 0 | | +1.41 | |

### *2.6. Analysis of Cell Growth and Determination of Glycerol and Metabolic Byproducts*

Ultraviolet-visible spectroscopy (600 nm) was used to measure the optical density (OD) of the 2GPP strain. The cells were dried at 70 °C for 24 h. Linear regression was performed to correlate dry cell weight with optical density, and the inoculum concentration ($g \cdot L^{-1}$) was calculated by Equation (1):

$$Biomass = 0.0325 \cdot OD - 0.0117 \quad (1)$$

The concentration of lactic acid, 1,3-propanediol, and glycerol were evaluated using high-performance liquid chromatography. This was performed with 300 × 7.8 mm Aminex HPX-87H column and 30 × 4.6 mm precolumn (Bio-Rad); mobile phase 0.005 $mol \cdot L^{-1}$ $H_2SO_4$; flow rate 0.6 $mL \cdot min^{-1}$; detector and column temperature 40 °C.

The optical purity of the produced lactic acid enantiomers was determined with a Diode-Array Detection (DAD) detector (254 nm), a Chirex 3126 (D)-penicillamine (150 × 4.6 mm; Phenomenex) column working at 30 °C, and a copper(II) sulfate 0.001 M solution as a mobile phase at a flow rate of 1.0 $mL \cdot min^{-1}$. A calibration curve was built using standard enantiomer samples with a retention time of 2.77 min for L-(+)-lactic acid and 9.75 min for D-(−)-lactic acid.

### *2.7. Fermentation Yield*

The substrate's bioconversion into the product ($g_{product} \cdot g_{glycerol}{}^{-1}$) was calculated using Equation (2) to obtain the fermentation yield [42].

$$Y_{P/S} = -\frac{dP}{dS} = -\frac{P_f - P_i}{S_f - S_i} \quad (2)$$

where $Y_{P/S}$ is the conversion factor, $P$ is the product concentration ($g \cdot L^{-1}$) given as the difference between the final ($P_f$) and initial ($P_i$) product concentrations obtained in the fermentation. $S_f$ and $S_i$ are the substrate's final and starting concentrations, respectively, while $S$ is the substrate concentration ($g \cdot L^{-1}$).

### *2.8. Proton Nuclear Magnetic Resonance*

$^{1}$H NMR spectroscopy was used to provide information on the production of lactic acid and 1,3-propanediol from the aliquots taken at the end of fermentation. The experimental runs were performed on a Bruker Magneto Ascend 600 MHz. Approximately 20 mg samples were dissolved in 1 mL of deuterated water ($D_2O$) containing tetramethylsilane (TMS) as the internal standard.

## 3. Results and Discussion

### *3.1. Selection of Bacteria in Microaerobiosis*

The eighteen obtained bacterial strains were evaluated for 48 h under microaerobiosis in 96-well microplates. This experiment aimed to evaluate the elite strain. It was observed that 17 strains did not produce a significant amount of lactic acid, nor did they significantly consume glycerol. It is known that the pyruvate pathway is favored under anaerobic conditions. As lactic acid is the final product of interest, fermentations were carried out with a sealed 96-well plate. The main challenge is to predominantly produce lactic acid, inhibiting the other products derived from the pyruvate metabolic pathway, such as 2,3-butanediol, 1,3-propanediol, and ethanol [9].

The 2GPP strain produced lactic acid and 1,3-propanediol, and consumed a significant amount of glycerol. After 48 h, 92.5% of the initial glycerol was consumed, and the bacteria metabolized 3.8 $g{\cdot}L^{-1}$ lactic acid and 5 $g{\cdot}L^{-1}$ 1,3-propanediol. The $Y_{LA/Gly}$ was 0.10 and $Y_{1,3\text{-}PDO/Gly}$ 0.14. Therefore, 2GPP was selected for identification.

Vivek et al. [43] investigated the efficiency of batch cultivation by *Lactobacillus brevis* with MRS medium, supplemented with 3% ($w{\cdot}v^{-1}$) glucose and 20 $g{\cdot}L^{-1}$ pure glycerol as a carbon source. Anaerobic batch fermentation was carried out at 37 °C and 200 rpm for 72 h. This experiment produced approximately 14.8 $g{\cdot}L^{-1}$ of 1,3-propanediol, 14 $g{\cdot}L^{-1}$ of lactic acid, and 13 $g{\cdot}L^{-1}$ of acetic acid, with a yield of 0.65 $g_{1,3\text{-PDO}}{\cdot}g_{Glycerol}{}^{-1}$, 0.61 $g_{LA}{\cdot}g_{Glycerol}{}^{-1}$, and a 0.56 $g_{AceticAcid}{\cdot}g_{Glycerol}{}^{-1}$ conversion rate.

### *3.2. Phylogenetic Analysis of the 2GPP Selected Strain*

According to the 16S rRNA partial gene sequence assay (1290 bp), the 2GPP is a member of the *Enterobacteriaceae* family with 100% identity with the *Klebsiella* genus. When the 2GPP 16S rRNA sequence was analyzed using the Blast tool and compared to the GenBank type strains' database, 99.77% identity was observed with the *K. pneumoniae* subsp. *rhinoscleromatis* strain R-70 (accession number NR_037084.1). The genetic relationships between the 2GPP strain from the Brazilian Cerrado soil and other *Klebsiella* species (type strains) were examined using the partial sequence of the 16S rRNA through Maximum likelihood (ML) phylogenetic inference. The phylogenetic analysis (Figure 2) showed that the 2GPP strain could be clustered in the *K. pneumoniae* species complex, including the species *K. pneumoniae*, *K. variicola*, *K. quasivariicola*, *K. africanensis*, *K. quasipneumoniae,* and others. As previously reported [44], the 16S rRNA region is often not different enough to separate closely related *Klebsiella* species, and other protein-coding genes (rpoB gene) must be analyzed to better assess the genetic relationship between species in the *K. pneumoniae* complex.

The *K. pneumoniae* complex includes at least seven closely related species [45], all of them gram-negative, encapsulated bacteria with facultative anaerobic metabolism. Although some species in this complex are known for their pathogenic potential, other species are observed in the literature with biotechnological potential. Niu et al. [14], Luo et al. [13], and Wei et al. [18] showed the potential of different strains of the *K. pneumoniae* complex, which could produce hydrogen, 3-hydroxypropionic acid, and 2-Ketogluconic acid, respectively.

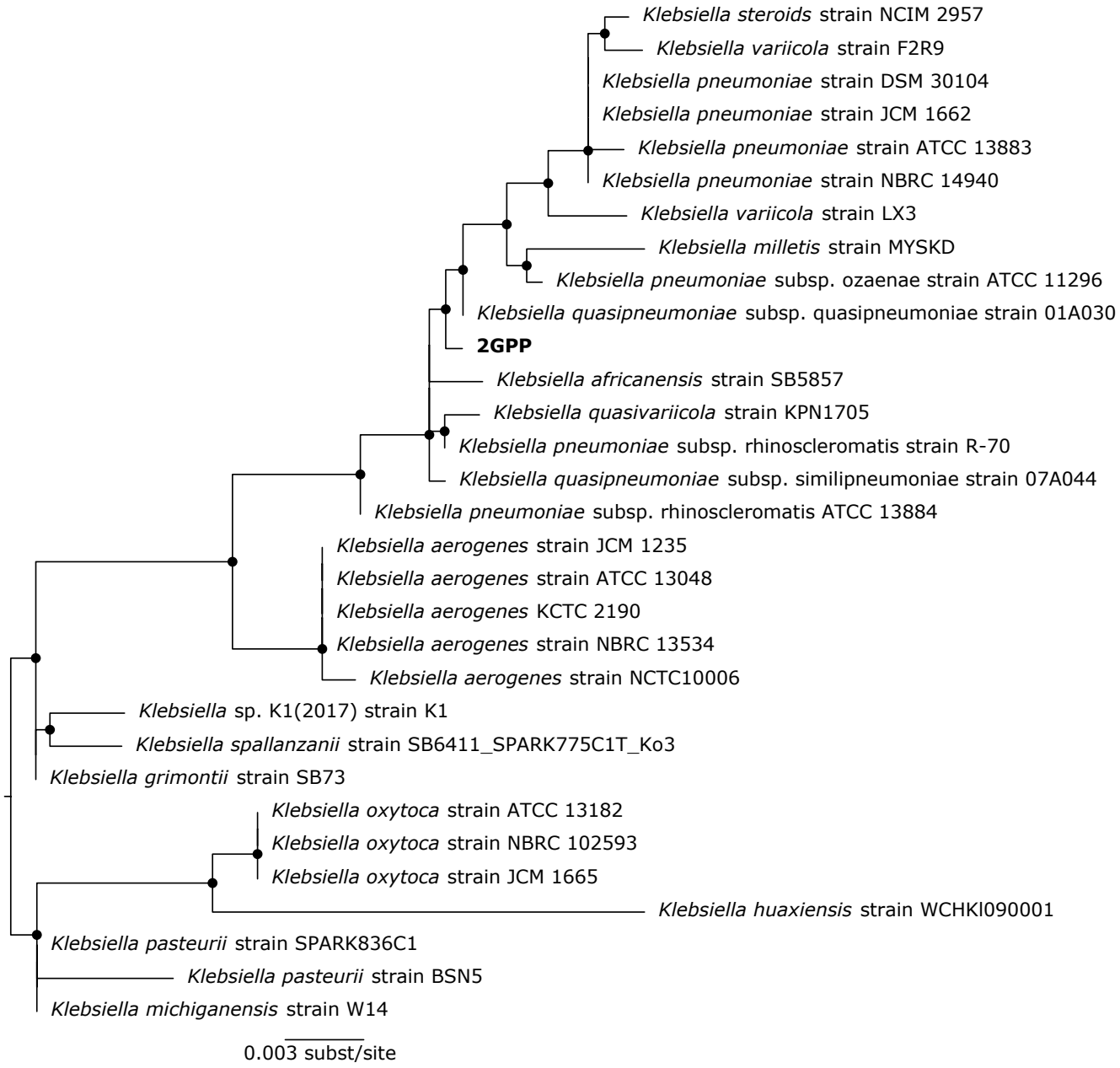


**Figure 2.** Maximum likelihood (ML) phylogenetic tree of 16S rRNA partial gene sequence (1290 pb) from 2GPP strain and 30 *Klebsiella* species/strains. The ML tree was inferred with PhyML, and the node support was estimated with the SH-aLRT test. Support values $\geq$ 100% SH-aLRT are displayed with black circles at nodes.

### *3.3. Evaluation of the Inoculum Concentration and Agitation in Lactic Acid Production by Klebsiella sp. 2GPP*

The experiment using a 15 mL flask aimed to increase the scale, maintaining the experiment's conditions, in 96-well microplates. To establish appropriate conditions to synthesize lactic acid from glycerol, we evaluated the effect of inoculum concentration. Table 4 shows lactic acid production at different bacterial cream concentrations in an M9 broth medium supplemented with 4% glycerol at concentrations of 5.0, 4.2, 2.0, and 1.0 $g \cdot L^{-1}$ for 48 h at 120 rpm at 32 °C. Another analyzed factor was agitation. A flask with 4.2 $g \cdot L^{-1}$ of inoculum was kept unstirred in an incubator to investigate this factor's influence on lactic acid production for 48 h at 32 °C.

Concerning the experiments performed with agitation at 120 rpm, the best stirring condition was 5 $g \cdot L^{-1}$, which produced 1.94 $g \cdot L^{-1}$ of lactic acid. The yield of lactic acid production ($Y_{LA/Gly}$) was 0.54, and the yield of 1,3-propanediol ($Y_{1,3\text{-}PDO/Gly}$) was 0.36. It can be observed that, in the experiments in which the cream was more diluted in the medium, the production of lactic acid was lower, since a lower number of cells metabolized glycerol and, consequently, less lactic acid was produced.

**Table 4.** Evaluation of the production of lactic acid by *Klebsiella* sp. 2GPP with glycerol as carbon source and variations in inoculum in a 15 mL flask.

| | 24 h | | 48 h | | | |
|---|---|---|---|---|---|---|
| **Inoculum Concentration ($g \cdot L^{-1}$)** | **Lactic Acid ($g \cdot L^{-1}$)** | **1,3-Propanediol ($g \cdot L^{-1}$)** | **Lactic Acid ($g \cdot L^{-1}$)** | **1,3-Propanediol ($g \cdot L^{-1}$)** | **$Y_{LA/Gly}$** | **$Y_{1,3\text{-}PDO/Gly}$** |
| 5.0 (120 rpm) | 1.00 ± 0.09 | 0.61 ± 0.09 | 1.94 ± 0.41 | 1.28 ± 0.20 | 0.54 | 0.36 |
| 4.2 (120 rpm) | 1.10 ± 0.13 | 0.87 ± 0.14 | 1.43 ± 0.21 | 1.09 ± 0.21 | 0.41 | 0.31 |
| 2.0 (120 rpm) | 0.79 ± 0.12 | 0.53 ± 0.11 | 1.34 ± 0,13 | 0.97 ± 0.27 | 0.38 | 0.28 |
| 1.0 (120 rpm) | 0.81 ± 0.19 | 0.68 ± 0.25 | 1.29 ± 0.13 | 0.94 ± 0.59 | 0.37 | 0.26 |
| 4.2 (unstirred) | 2.29 ± 0.02 | 1.48 ± 0.01 | 2.88 ± 0.39 | 1.91 ± 0.27 | 0.81 | 0.55 |

Oh et al. [46] carried out a fed-batch fermentation with *K. pneumoniae* and concluded that the inoculation of cells at 4.8 $g \cdot L^{-1}$ resulted in a reduced production of 1,3-propanediol. The fed-batch with 3.2 $g \cdot L^{-1}$ of inoculum concentration produced 81.1 $g \cdot L^{-1}$ of 1,3-propanediol. In this experiment, it was possible to infer the opposite: higher inoculum concentrations produced more 1,3-propanediol.

Another analyzed factor was agitation. When fermentation was performed in a flask in an incubator without an agitation, there was less oxygen, which favors the bacteria's anaerobic pathway, corroborating with Zhang et al. [20], who suggested that anaerobic conditions favor the production of 1,3-propanediol and pyruvate. The same can be verified by comparing the same inoculum concentration (4.2 $g \cdot L^{-1}$) with and without agitation. The experiment performed in the incubator and without agitation showed a lactic acid production that was twice as high as the one produced in the rotary shaker experiment at 120 rpm, as shown in Table 4.

In this experiment, it was inferred that the reductive and oxidative anaerobic metabolisms occurred simultaneously. The biomass and lactic acid are generated by the reductive metabolism of glycerol while 1,3-propanediol is generated by oxidative metabolisms. The production of only two final products is desirable. The next experiments were performed to minimize the synthesis of 1,3-propanediol and increase lactic acid production [20].

In all fermentations, there was a decrease in glycerol concentration. However, glycerol consumption was higher in the experiment without aeration, showing that the lower the amount of oxygen in the medium, the more glycerol will be metabolized for lactic acid production. This result corroborates the idea that the bacteria produce more of the product of interest under anaerobic conditions. About 93% of glycerol was consumed in the fermentation with 4.2 $g \cdot L^{-1}$ of inoculum without agitation.

Table 4 shows that the production of 1,3-propanediol was lower than in the mini-reactor experiment. This result is desirable, as the product of interest is lactic acid. The production behavior of 1,3-propanediol is similar to that of lactic acid; the fewer the incubated cells, the lower the production. Additionally, experiments without agitation have a higher production of 1,3- propanediol. Rossi et al. [16] showed that, under anaerobiosis, *K. pneumoniae* produces more 1,3-propanediol (6.88 $g \cdot L^{-1}$).

Considering the experiment with the highest lactic acid bioconversion, the yield of lactic acid ($Y_{LA/Gly}$) was 0.81, and the yield of 1,3-propanediol ($Y_{1,3\text{-}PDO/Gly}$) was 0.55. The yield of 1,3-propanediol was similar to that obtained by Zhang et al. [47], which resulted in yields between 0.32 to 0.54 through *Clostridium butyricum* batch fermentation. Hong et al. [48] achieved a yield ($Y_{LA/Gly}$) of 0.9 for lactic acid using *E. coli* AC-521 fed-batch fermentation.

As 1,3-propanediol and lactic acid compete for the same substrate (glycerol), to improve lactic acid bioconversion, experimental conditions were analyzed to minimize the production of 1,3-propanediol. This was a challenge, since a preferential and simpler route produces 1,3-propanediol. In addition, the separation of compounds is not trivial and generates more costs [9].

$^{1}$H NMR was used to reaffirm the production of lactic acid and 1,3-propanediol as the main metabolites at the end of fermentation. After microaneorbiosis fermentation with 2GPP, the spectrum represented in Figure 3 was obtained. The signals of L-(+)-lactic acid and 1,3-propanediol were assigned. Signals "a", "b" and "c" are related to the lactic acid molecule. The hydrogen of the chiral carbon is characterized by the chemical shift of 4.11 ppm, indicated by "a". Methyl group was indicated by the "b" signal in doublet form at 1.14 ppm. The hydroxyl hydrogen was assigned to the doublet indicated by "c" at 3.55 ppm. Signals "d", "e" and "f" were assigned to the 1,3-propanediol molecule. The hydrogen indicated by the letter "d" is a quintuplet with a chemical shift of 1.33 ppm. The triplet "e" at 3.78 ppm indicates two-carbon hydrogens with the same chemical environment. The hydroxyl hydrogens were assigned to the triplet indicated by "f" at 3.65 ppm.

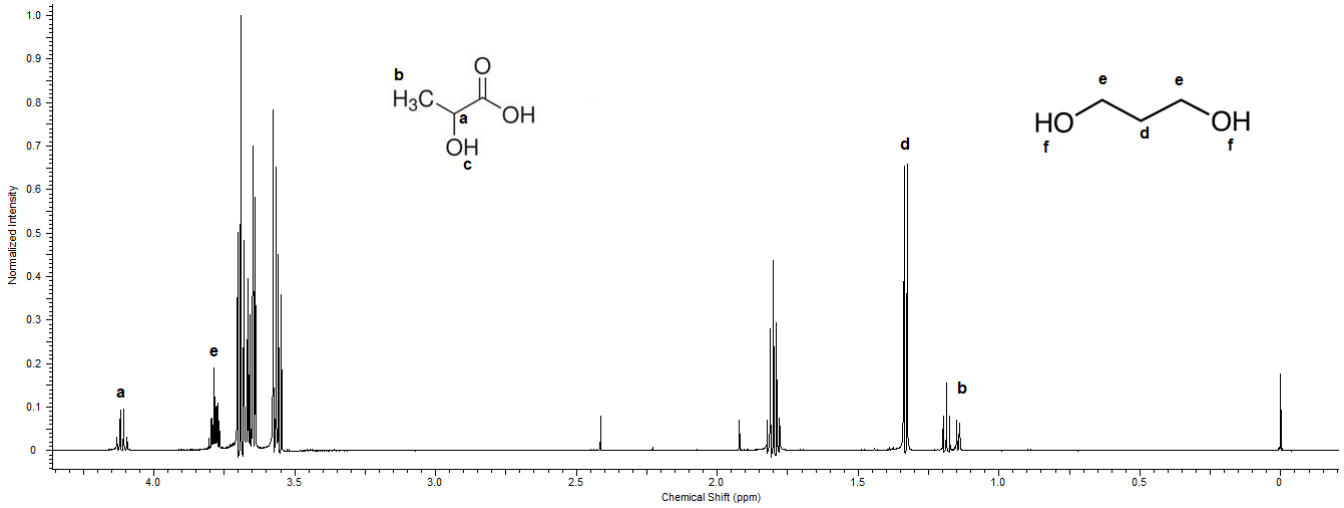


**Figure 3.** $^{1}$H NMR spectrum related to the end of fermentation containing lactic acid and 1,3-propanediol.

In the present work, HPLC measurements were carried out to provide insight into the production of lactic acid enantiomers. According to Figure 4, the 2GPP strain was able to produce L-(+)-lactic acid with optical purity (retention time of 2.77 min). From the chromatogram obtained for the pure enantiomer samples, the retention time for L-(+)-lactic acid and D-(−)-lactic acid was expected to be equal to approximately 2.70 min and 9.75 min, respectively (Figure 4A). As depicted in Figure 4B, only the L-isomer was produced, since no signal was detected at about 9.8 min. The signals detected at retention times within the interval from approximately 1.0 min to 2.6 min were related to other species in the fermentation medium. This finding differs from the work developed by Sangproo et al. [49]. According to this study, wild-type *Klebsiella oxytoca* produced approximately 2.7 $g \cdot L^{-1}$ of D-(-)-lactate during glucose fermentation, reaching a yield of 0.12 $g \cdot g^{-1}$ and productivity of 0.11 $g \cdot L^{-1} \cdot h^{-1}$ after 72 h, along with ethyl alcohol, 2,3-butanediol, succinate, acetate and formate.

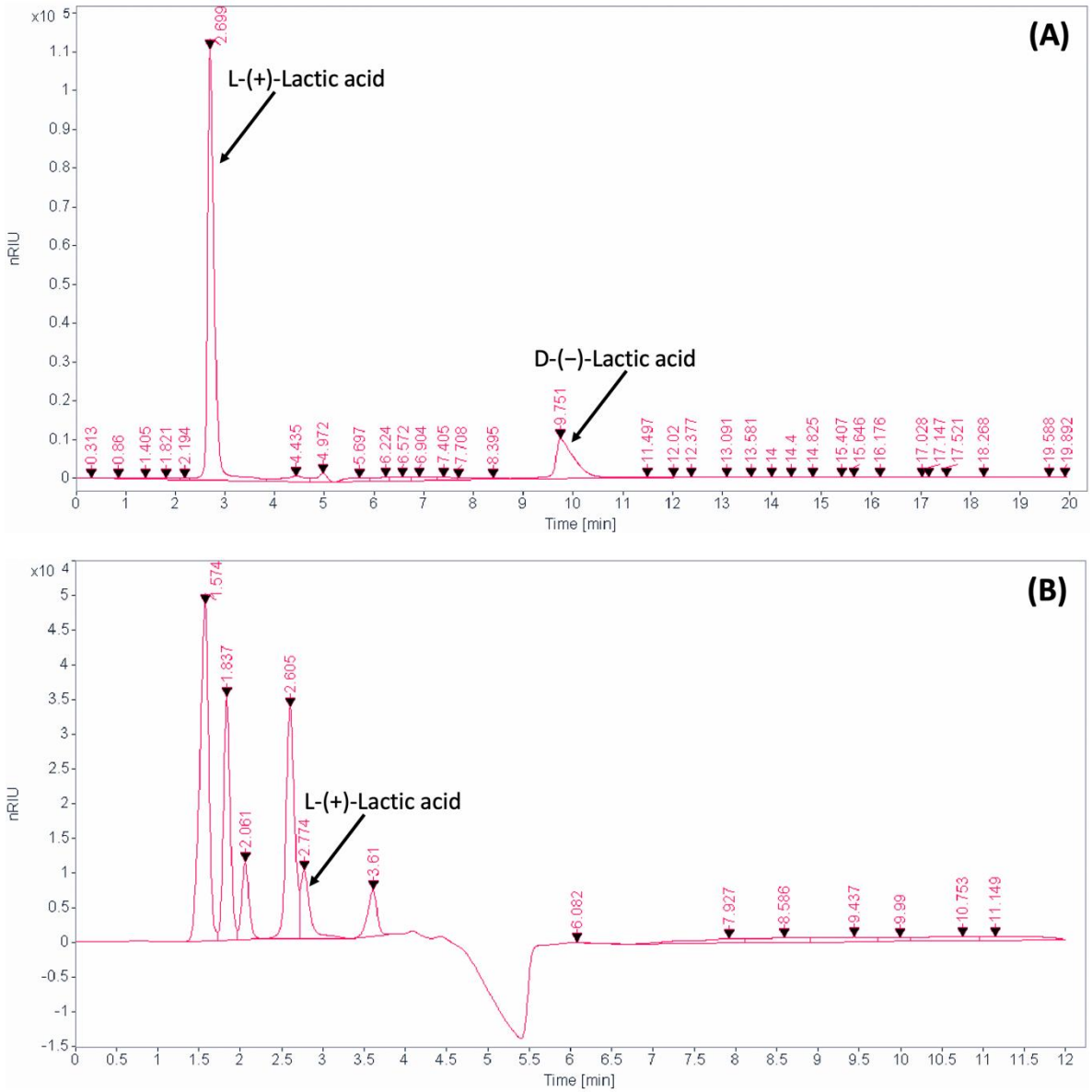


**Figure 4.** HPLC chromatogram of (**A**) standard samples of L-(+)-Lactic acid and D-(−)-Lactic acid enantiomers, and (**B**) fermentation medium by using *Klebsiella* sp. 2GPP with glycerol as a carbon source.

### *3.4. $2^3$ Factorial Design*

Figure 5 shows the lactic acid and 1,3-propanediol obtained in the experiments using an M9 medium supplemented with 10% glycerol. The three variables were chosen as observed and experimentally. Agitation was maintained at 50 rpm, as the bacterium *Klebsiella* sp. 2GPP is facultatively anaerobic and has obtained better results for lactic acid production with smaller amplitudes, leading to less oxygenation [15,18].

The significance of the effects on temperature, inoculum concentration, and pH was evaluated for experiments using glycerol as a carbon source, based on analysis of variance (ANOVA) using the Statistica program as a tool. Based on the Pareto diagram for standardized effect estimates concerning lactic acid production, considering the equivalent of 48 h of fermentation in glycerol experiments, only temperature and the nonlinear effect of curvature ($d_{curv}$, corresponds to a quadratic contribution of experimental runs performed at the central point of the experimental planning) are significant for lactic acid concentration.

The ANOVA test indicated that the confidence level for these analyzes was 95% ($p$ = 0.05), as depicted in Figure 6. The correlation coefficient ($R^2$) was equal to 96.4%.

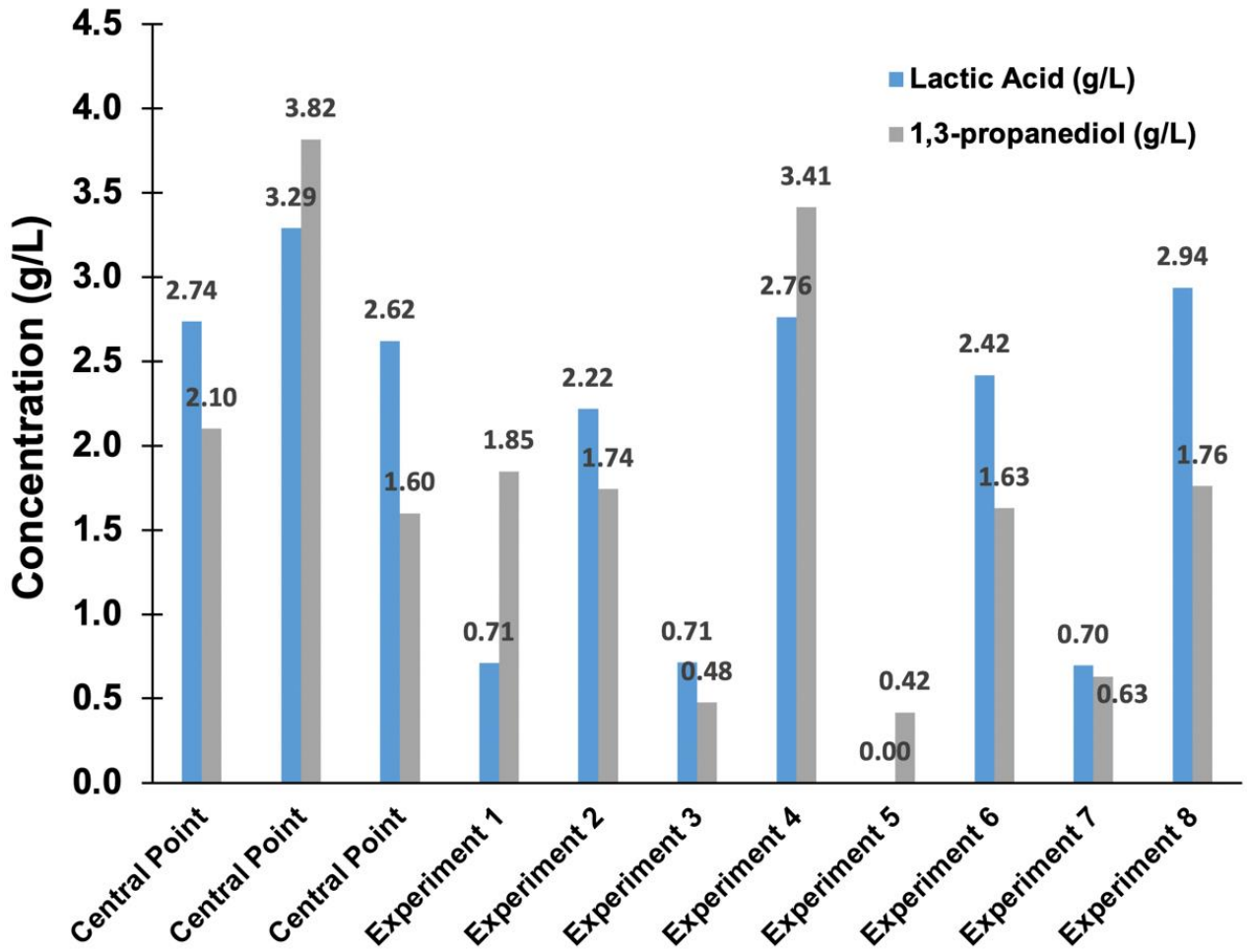


**Figure 5.** Production of lactic acid and 1,3-propanediol obtained in M9 medium with 10% glycerol for 48 h for the conditions related to the factorial design experiments. The central point corresponds to Experiments 9, 10, and 11, respectively, described in Table 3.

As the curvature corresponds to a nonlinear behavior between factor(s) and the response variable(s), for any $2^k$ experimental design, a check for curvature should be considered a *sine qua non* to evaluate the linearity between the variables, because it provides significant insights into the process features, allowing for an appropriate choice of new experimental planning strategies that allow for the use of second-order models. $\delta_{curv}$ is generally expressed as a quadratic contribution at the midpoint of the experimental design, given as [50–52]:

$$\delta_{curv} = \begin{cases} 1, \text{ if the point is a center point} \\ 0, \text{ otherwise} \end{cases} \quad (3)$$

By considering only the significant variables, the fermentation process model with glycerol can be represented according to Equation (4).

$$Y = 1.75 + 0.83 \cdot X_1 + 1.13 \cdot \delta_{curv} \quad (4)$$

Equation (4) clearly shows how important the curvature effect is, and unequivocally indicates the presence of critical, statistically significant nonlinear effects, considering the order of magnitude of the estimated parameters.

By analyzing the level curves (Figure 7), it is possible to verify that there is a tendency towards higher lactic acid production when the temperature ($X_1$) and pH increases ($X_3$) may represent higher lactic acid production. Inoculum concentration was not significant. Ji et al. [53] obtained excellent results with fermentations at 37 °C for *K. pneumoniae* and verified that, when pH was alkaline, the predominant form in the fermentation broth is the lactic acid conjugate base (lactate), and lactate is less toxic to the bacterial cell. They also concluded that an intrinsic mechanism of bacteria self-protection should cause the exchange of metabolic pathways to adapt to the environmental variations in the pH of *K. pneumoniae*.

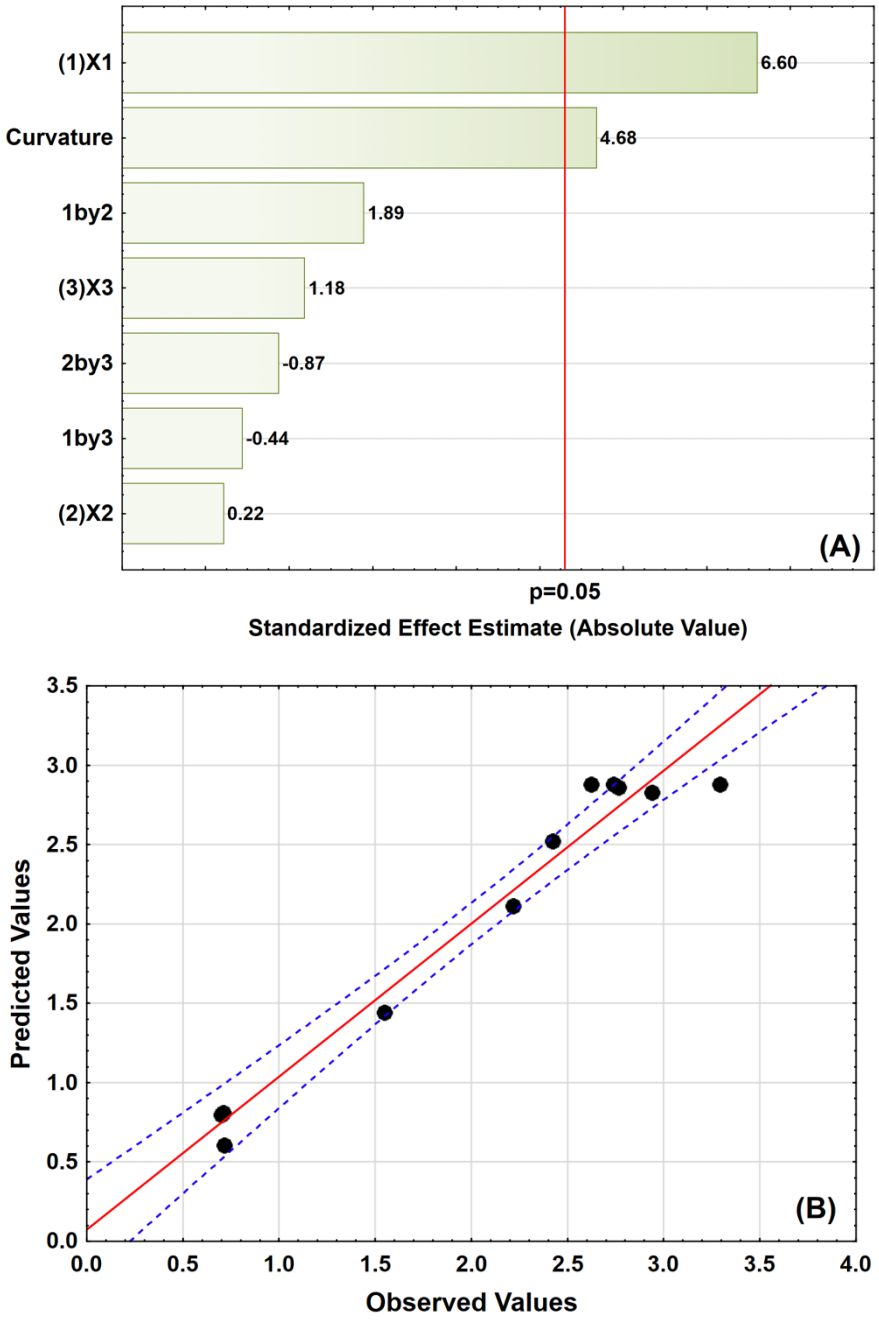


**Figure 6.** (**A**) Pareto chart for standardized effects estimative of the temperature ($X_1$), Inoculum concentration ($X_2$), and pH ($X_3$) and (**B**) prediction of the regression model for experimental data for the production of lactic acid (N.B. linear fit: $y = 0.0741 + 0.964 \cdot x$). The dashed lines stand for the regression bands built with a 95% confidence level.

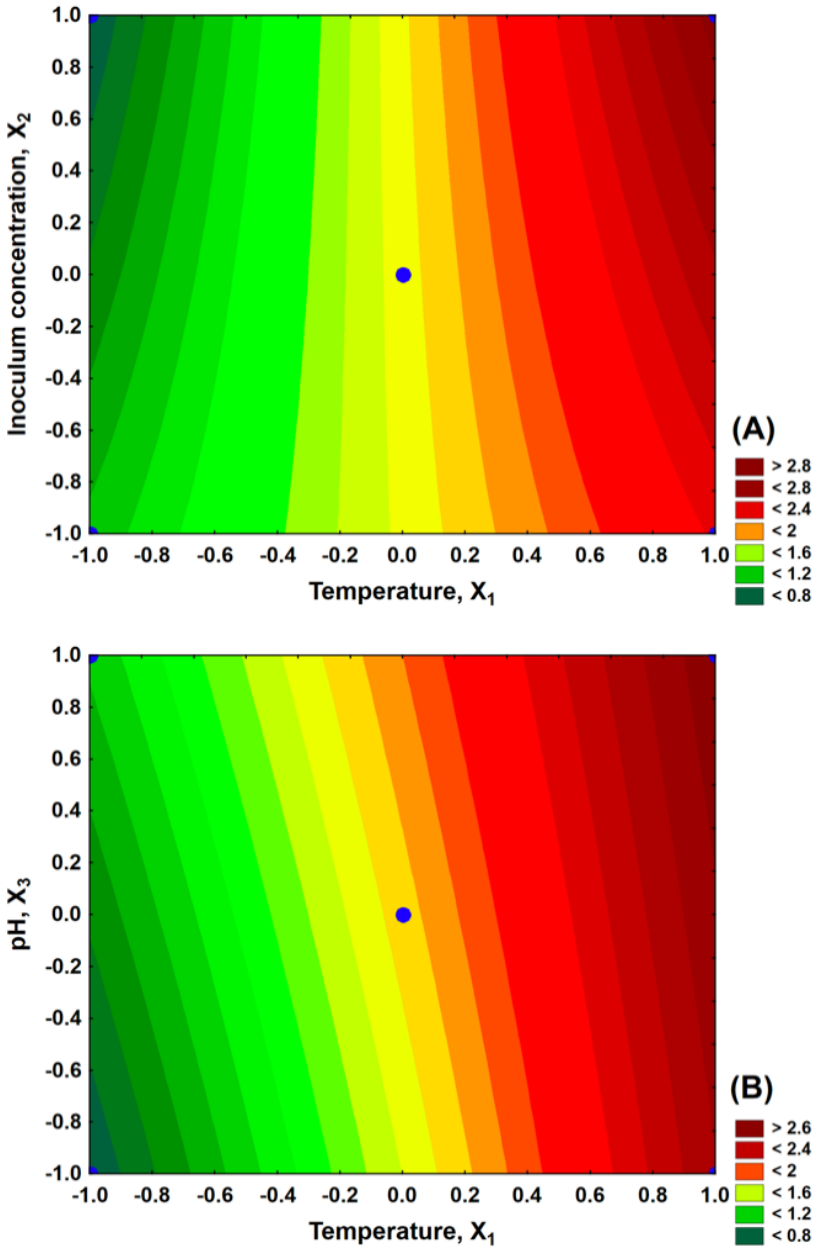


**Figure 7.** Contour plots for (**A**) temperature ($X_1$) versus inoculum concentration ($X_2$), and (**B**) temperature ($X_1$) versus pH ($X_3$) for the production of lactic acid.

According to research by Oh et al. [46], the maximum production of 1,3-propanediol was achieved in the fed-batch fermentation with *K. pneumoniae* and pH = 6.5. Ji et al. [53] also verified that lactic acid formation was inhibited in pH 6.3 under similar conditions. The results obtained in this experiment corroborate this observation and emphasize the importance of pH to improve a specific compound's production. This study modified process variables to influence bacteria to synthesize lactic acid instead of 1,3-propanediol by a non-preferred route, producing 3.29 $g \cdot L^{-1}$ of lactic acid and a yield ($Y_{LA/Gly}$) of 0.55. The pH adjustment proved to be a crucial factor in the increased production of lactic acid. However, the production of 1,3-propanediol did not decline and yielded a $Y_{1,3\text{-PDO/Gly}}$ 0.64.

Rossi et al. [16], using the anaerobic fermentation of *K. pneumoniae*, produced 1 $g \cdot L^{-1}$ lactic acid, 5 $g \cdot L^{-1}$ ethanol, and 20 $g \cdot L^{-1}$ 1,3-propanediol metabolizing residual glycerol. Comparing these results with this experiment, we can note that it was possible to decrease the production of 1,3-propanediol, which is the preferred route. In addition, the production of lactic acid increased.

### *3.5. Rotatable Central Composite Design*

This experiment considered the previously obtained results, which indicated that higher pH values and temperatures could minimize the production of 1,3-propanediol. The variables of this experiment were chosen from the most promising variables in the $2^3$ factorial design. Figure 8 shows how lactic acid and 1,3-propanediol were obtained in the experiments using an M9 medium supplemented with 4% glycerol.

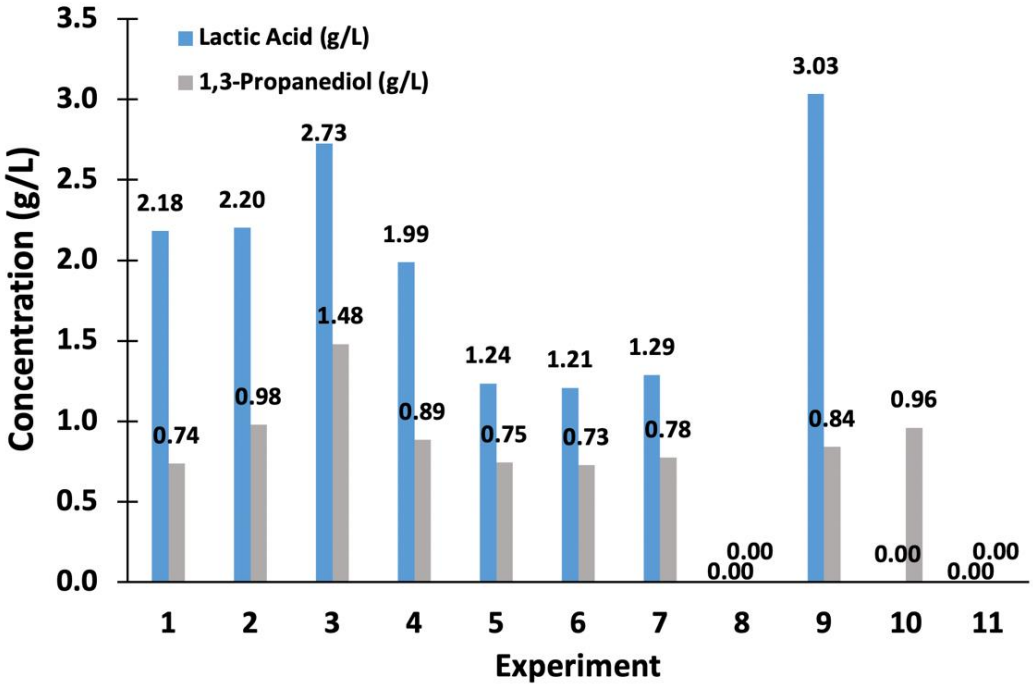


**Figure 8.** Production of lactic acid and 1,3-propanediol obtained in M9 medium with 4% glycerol for 72 h was related to the rotational composite central design experiments.

It was possible to verify that, with the experiments' design, there was a higher production of lactic acid and a reduced production of 1,3-propanediol. The significance of the effects of inoculum concentration and pH were evaluated for the experiments presented in Table 3 using glycerol as a carbon source, based on analysis of variance (ANOVA) using the TIBCO Statistica program as a tool.

The Pareto diagram for standardized effects concerning lactic acid production (Figure 9A) indicates that inoculum concentration and pH are significant for lactic acid production and satisfy the evaluated range's statistical hypothesis. The ANOVA test showed that the inoculum concentration effect was significant for lactic acid production. The effect of the quadratic ratio of pH and inoculum concentration was also significant, as was the effect of the ratio of inoculum concentration to pH. The confidence level for these analyzes was 95% ($p = 0.05$). Figure 9B represents the model's ability to predict glycerol consumption, indicating that the regression model satisfactorily predicts the experimental data. The regression model for experiment assays using glycerol as a carbon source with a correlation coefficient ($R^2$) of 92.08% can be expressed as:

$$Y = 45.07 - 3.33 \cdot X_1^2 - 4.48 \cdot X_2^2 \quad (5)$$

The results corroborate those obtained in the Pareto diagram, indicating that higher inoculum concentrations tend to produce more lactic acid. When analyzing the pH factor, there is a greater tendency towards production near the central point, indicating that the medium must have a pH of 9.5. Ji et al. [53] observed that, at a pH from 7.1 to 8.0, the dominant byproduct was lactic acid in fed-batch fermentation by *K. pneumoniae*.

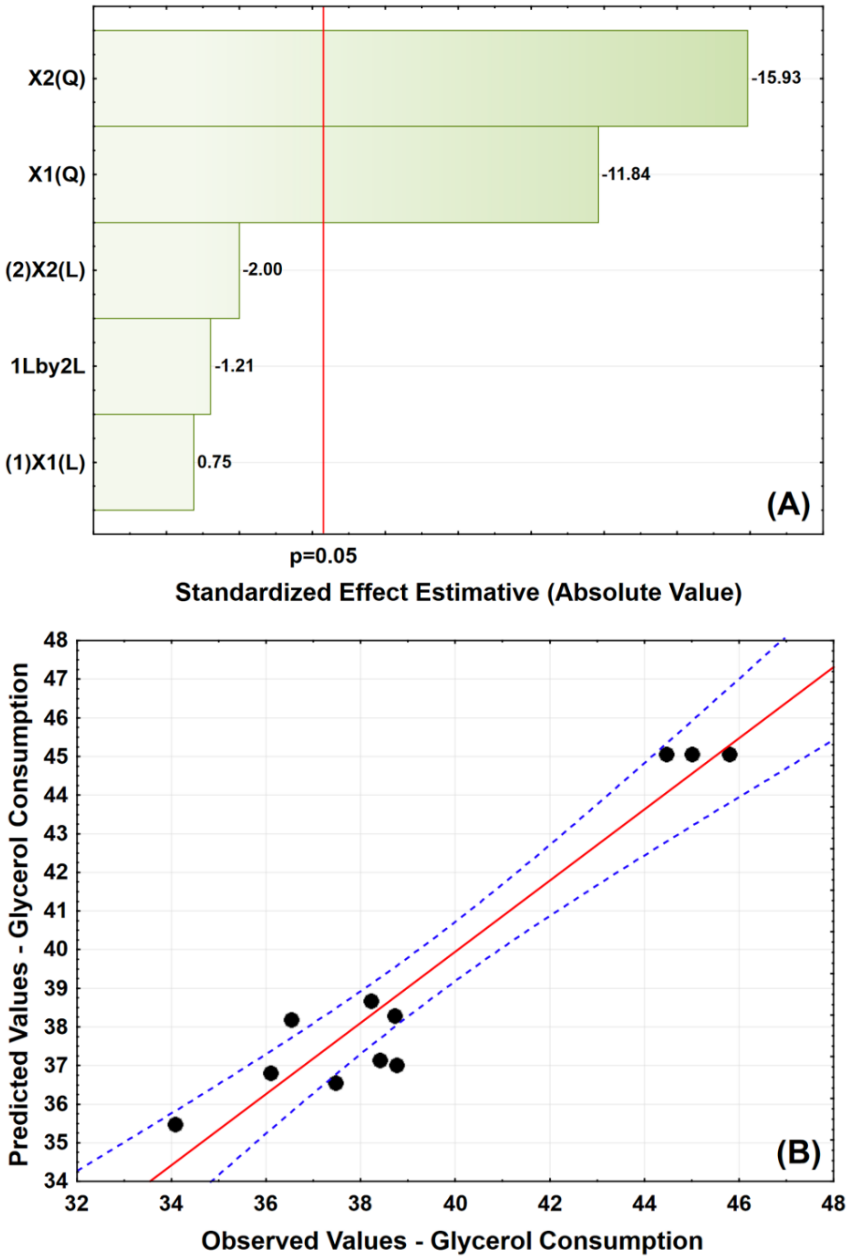


**Figure 9.** (**A**) Pareto chart for standardized effect estimative of the inoculum concentration ($X_1$) and pH ($X_2$) and (**B**) prediction of the regression model for experimental data for the glycerol consumption (N.B. linear fit: $y = 3.1195 + 0.9208 \cdot x$). The dashed lines stand for the regression bands built with a 95% confidence level.

With the surface graph and contour plot shown in Figure 10A,B, it is possible to verify the point of maximum glycerol consumption. This fact shows that higher glycerol consumption conditions are the central point, and are also the best conditions for the production of lactic acid. Fermentation was carried out for 72 h, reaching the theoretical point of the maximum consumption of glycerol, making longer fermentations unnecessary.

It was possible to find the point of the maximum consumption of glycerol, maximize the production of lactic acid and significantly reduce the production of 1,3-propanediol with the experimental design. Compared to the $2^3$ factorial design experiment, lactic acid production remained close to 3 $g \cdot L^{-1}$, and the production of 1,3-propanediol dropped to 0.8 $g \cdot L^{-1}$, showing an evident improvement that was only achieved by modifying simple

process variables. It is worth mentioning that it was possible to induce bacteria to produce lactic acid, a non-preferred route.

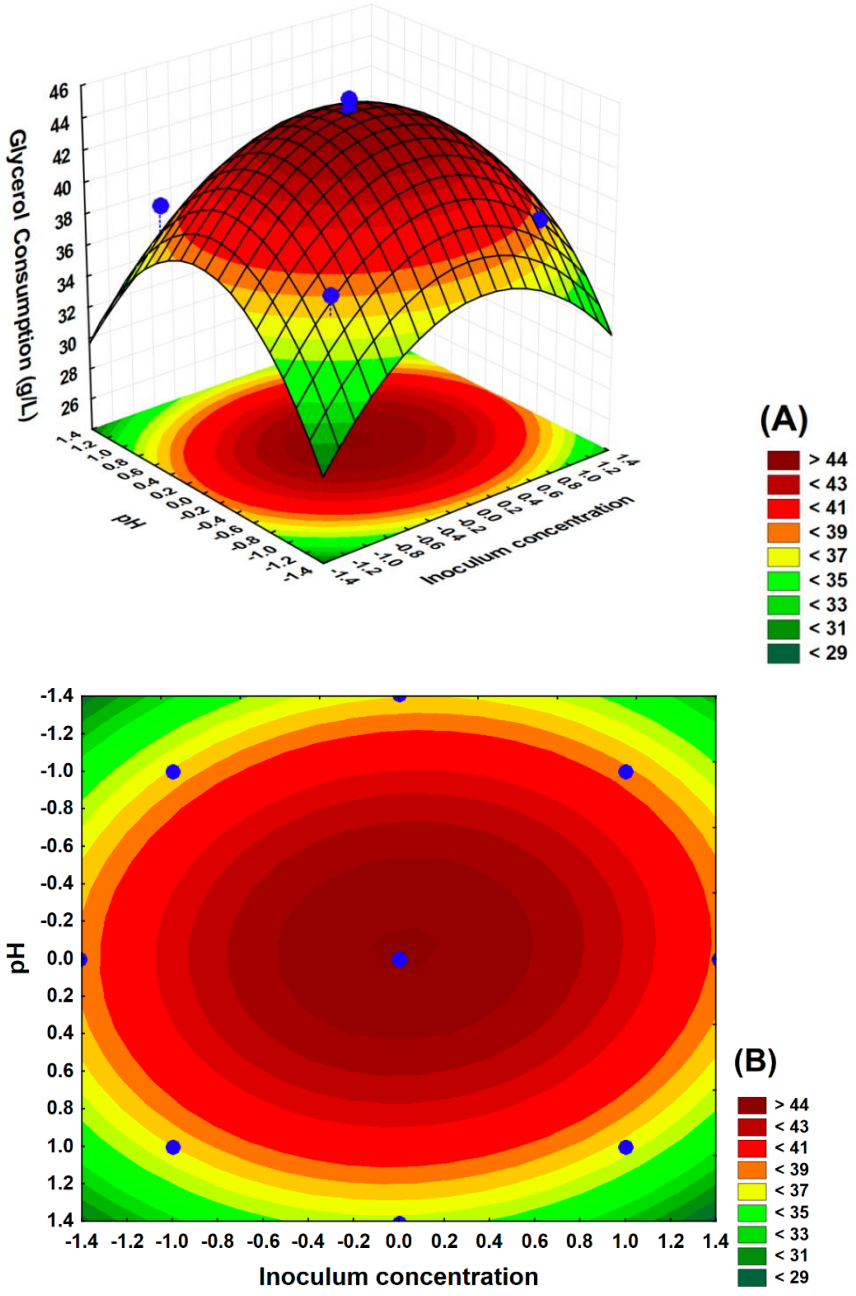


**Figure 10.** Surface plot for glycerol consumption for rotatable central composite design (**A**) experiments and contour plot (**B**) for glycerol consumption for experiment inoculum concentration versus pH.

In this experiment, the yield was $Y_{LA/Gly}$ 0.68, higher than the $2^3$ factorial design. For 1,3-propanediol, the lowest yield value was achieved among the experiments: $Y_{1,3\text{-PDO/Gly}}$ 0.18. Ji et al. [53] obtained yield values of 0.04 and 0.58 for lactic acid and 1,3-propanediol, respectively, in a fed-batch fermentation by *K. pneumoniae.*

According to previous study developed by Wang et al. [54], *K. pneumoniae* produced about 12 $g \cdot L^{-1}$ of lactic acid in bioreactors. There was also the production of 1,3-propanediol, 2,3-butanediol, 3-hydroxypropionic acid and acetic acid. In the present study, it was possible to produce only lactic acid and 1,3-propanediol, aiding in future purifications.

Solvent extraction is a technique normally used to purify lactic acid. Chen et al. [8] studied the recovery of the L-(+)-lactic acid obtained by fermentation. A multiple-pass distillation was performed after the solvent extraction to recover L-lactic acid with a purity of 91.3% and a yield of 74.63%.

The experiment with the highest yield was the one that verified the evaluation of the inoculum concentration and agitation, performed in 15 mL flasks ($Y_{LA/Gly}$ = 0.81). Thus, it is possible to deduce that higher yields can be achieved from changes in fermentation conditions. This study can be continued in bioreactors to obtain even more promising results, starting from a wild-type bacterium and analyzing other process variables, such as substrate feed, temperature control, and nitrogen addition.

## 4. Conclusions

The present work can verify that it is possible to produce high-value products from fermentative processes using glycerol as a substrate. The selected strain, 2GPP, was identified as a *K. pneumoniae* complex and has also been shown to produce L-(+)-lactic acid with optical purity. The influence of temperature, pH, and inoculum concentration on lactic acid production and the growth in *Klebsiella* sp. 2GPP was studied in 125 mL conical flasks. The experimental design indicated a tendency to maximize lactic acid production and increase glycerol consumption at 37 °C and pH 9.5. A yield of 0.81 was achieved for the metabolite of interest, lactic acid.

Although the metabolite purification process remains a challenge, production via fermentation has the main advantage of enabling the stereo-specific enantiomer of lactic acid, since the chemical route produced both isomers.

Finally, it was possible to obtain a wild-type microorganism that produced more lactic acid and minimized 1,3-propanediol production by changing factors such as temperature, pH, and inoculum concentration.

**Author Contributions:** The manuscript was written with the contributions of all authors. Conceived and designed the experiments: R.G.M.R.B. and J.R.C.L., analyzed the data: R.G.M.R.B., J.R.C.L., L.C.L.F., F.M. and S.B.G.; wrote the paper: R.G.M.R.B., J.R.C.L., L.C.L.F., F.M. and S.B.G. All authors have read and agreed to the published version of the manuscript.

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