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ORIGINAL ARTICLE

Xylonic Acid Production from Sugarcane Bagasse Hydrolysate Using an Engineered *Komagataella phaffii* Strain

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Abstract

Xylonic acid is an oxidized derivative of xylose that is used by the food, pharmaceutical, and chemical industries. It can be produced either microbially or chemically. Biological production is advantageous because of its safety, eco-friendliness, and mild process conditions. This study used a recombinant *Komagataella phaffii* strain modified to produce xylonic acid from xylose. Glucose and glycerol were evaluated as co-substrates in batch and fed-batch fermentations in flasks and in bioreactor cultures. Glycerol was an effective carbon source for improving growth and product production. Batch fermentations with glycerol produced 24.88 ± 1.64 g.L^{-1} of xylonic acid, with a yield of 0.55 ± 0.03 $g_{\text{xylonic acid}}.g_{\text{xylose}}{}^{-1}$ and a productivity of 0.26 ± 0.02 $g_{\text{xylonic acid}}.\text{L}^{-1}.\text{h}^{-1}$, while those with glucose yielded 20.30 ± 0.31 g.L^{-1}, with a conversion factor of 0.50 ± 0.01 $g_{\text{xylonic acid}}.g_{\text{xylose}}{}^{-1}$ and a productivity of 0.21 $g_{\text{xylonic acid}}.\text{L}^{-1}.\text{h}^{-1}$. Further, flask fermentations were run where glucose or glycerol was pulse fed. Glycerol-fed cultures produced 20.87 ± 0.30 g.L^{-1} of xylonic acid, with a productivity of 0.11 $g_{\text{xylonic acid}}.\text{L}^{-1}.\text{h}^{-1}$. Xylonic production was further improved in computer-controlled bioreactor cultures. Pulse batch fermentation (240 h, pH 5.5, 0.8 L.min^{-1} airflow) achieved a xylonic acid titer of 55.11 ± 2.36 g.L^{-1} with a productivity of 0.19 ± 0.01 $g_{\text{xylonic acid}}.\text{L}^{-1}.\text{h}^{-1}$. Finally, xylonic acid was produced from xylose using hydrolysate produced from sugarcane bagasse. The final concentration and productivity were 42.30 g.L^{-1} of xylonic acid and 0.44 g.L^{-1}.h^{-1}, respectively.

Keywords Xylonic acid · *Komagataella phaffii* · Hydrolysate · Sugarcane bagasse

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Introduction

Sugarcane bagasse is a renewable, abundant, and cost-effective feedstock rich in sugars. Approximately 279 million metric tons of bagasse are made available from sugarcane processing (1.6 billion tons per year). Its high carbohydrate content (44% cellulose, 28% hemicellulose, 21% lignin, 5% ash, and 2% extractives) makes it amenable for processing to biofuels and bioproducts. However, converting the carbohydrates to fermentable sugar requires pretreatment followed by enzymatic saccharification [1, 2].

Interest in organic acids derived from sugars has surged due to their potential in chemical synthesis and as polymer building blocks from biomass. They are extensively utilized in solvents, polyesters, polyamides, plasticizers, and food preservatives [3, 4]. Xylonic acid is notable for its thermal stability, biodegradability, and diverse potential applications [5]. It can enhance the flavor and shelf life of food products and serve as a precursor to chemicals like 1,2,4-butanetriol, used in polymer production. In the pharmaceutical sector, it functions as an antibiotic and a vitamin C metabolite [5–7]. Xylonic acid can replace gluconic acid, a crucial building block, with the added benefit of being derived from the non-food source xylose [1, 8]. Moreover, its potential high market value can bolster the bioethanol production process in biorefineries, improving the overall economic viability of the process [6].

Xylonic acid can be produced via chemical/electrochemical, enzymatic, and microbial biotransformation methods. Microbial biotransformation is the most economical and eco-friendly method and can utilize fungi, yeast, or bacteria [9].

Currently, xylonic acid commercial manufacturing is still in the developmental stage; however, microbial fermentation has emerged as the most promising and well-characterized production route, with substantial potential for optimization at an industrial scale [6].

Xylonic acid is oxidized biochemically from xylose by xylose dehydrogenase [6]. Microbes studied for its production include *Erwinia*, *Enterobacter cloacae*, *Gluconobacter oxydans*, *Pseudomonas fragi*, *Pseudomonas sacchari*, *Klebsiella pneumoniae*, and *Pichia kudriavzevii* [8, 10]. In a batch process using xylose as substrate, *Enterobacter cloacae* demonstrated the highest xylonic acid production, achieving 190 $g.L^{-1}$, with a yield of approximately 1 $g.g^{-1}$. *Gluconobacter oxydans NL71* also proved to be an effective natural producer, generating 109 $g.L^{-1}$ of xylonic acid in a batch process with a yield of 1.10 $g.g^{-1}$ [10]. Moreover, the highest concentration of xylonic acid (586.3 $g.L^{-1}$) was reported by Zhou et al. (2015). They used *G. oxydans NL71* in a pressurized bioreactor with high oxygen tension or a COS-SSRT (compressed pure oxygen gas with sealed stirred bioreactor) [11]. This system significantly improved the efficiency of xylose bioconversion, highlighting the need for high oxygen transfer rates to accelerate xylose oxidation by *G. oxydans* [5].

The need for robust industrial strains has driven the development of recombinant microorganisms, which are engineered by expressing xylose dehydrogenase [10]. Metabolic engineering optimizes microbial pathways to improve xylose uptake and boost product yields. Engineered strains can tolerate higher substrate levels, avoiding inhibition and enabling concentrated processing. By removing competing pathways, metabolic flux is redirected toward xylonic acid, reducing byproducts. These strains also adapt better to industrial bioreactors, making production scalable and cost-efficient [6, 12].

The first microbe engineered to oxidize xylose to xylonic acid (3.8 $g.L^{-1}$) was *Saccharomyces cerevisiae* by expressing the xylose dehydrogenase gene, XYD1, from *Trichoderma reesei* [6]. *Saccharomyces cerevisiae* with the xylB gene from *Caulobacter crescentus* produced 17 $g.L^{-1}$ of xylonic acid. Yield was improved by deleting xylose reductase activity

(encoded by GRE3), which converts xylose to xylitol, and expressing the xylC gene from *Caulobacter crescentus*. This strain produced 43 $g.L^{-1}$ of xylonic acid with a yield of 0.8 $g.g^{-1}$ [12]. The yeast *Pichia kudriavzevii* has also been engineered to produce xylonic acid by expressing the xylB gene from *Caulobacter crescentus.* Product productivity was especially sensitive to fermentation pH. At pH 5.5, the final concentration, yield, and productivity were 171 $g.L^{-1}$, 1.0 $g.g^{-1}$, and 1.4 $g.L^{-1}.h^{-1}$, respectively. At an acid pH (3), the amount produced fell to 146 $g.L^{-1}$ and a productivity of 1.2 $g.L^{-1}.h^{-1}$; yield remained unchanged [13].

Notably, the maximum reported xylonic acid production yields were achieved using synthetic media. Defined media are preferred to complex media because they allow for individual optimization of macro- and micro-nutrients and simplify product recovery. Inorganic sources of nitrogen are also less expensive than complex sources. Consequently, synthetic media are an appropriate standard when evaluating lignocellulosic hydrolysates in fermentation processes [10, 14].

The Microbial Genetics and Biotechnology Laboratory at Embrapa Agroenergia (Brazil) recently engineered *Komagataella phaffii* to produce xylonic acid from xylose. This yeast was selected because it grows at low pHs, achieves high cell densities, and standard molecular tools are available [15]. In this study, 11 putative bacterial and fungal xylose dehydrogenases were overexpressed. The resulting recombinant strains, carrying genes from bacteria (xylB-BS, xylB-HL, xylB-AM, xylB-MN, where BS, HL, and so on represent the abbreviation of the bacterial name) and fungi (XYD1-FM and XYD1-CO), successfully produced the acid. In a defined medium supplemented with xylose, the *K. phaffii* strains expressing bacterial xylose dehydrogenases produced the highest concentration and yields of xylonic acid: 23.0 to 37.0 $g.L^{-1}$ and 0.59 to 0.96 $g.g^{-1}$. The strain kXDH-HL, which expresses the XDH from *Halomonas lutea*, was the best performing yeast and produced 11.7 $g.L^{-1}$ of xylonic acid with a yield of 0.43 $g.g^{-1}$ using sugarcane bagasse hydrolysate [7, 10]. As *K. phaffii* does not metabolize xylose, the strain was fed glucose or glycerol as a carbon source [7].

Glycerol is of special interest because other studies have demonstrated that various *K. phaffii* strains can effectively employ crude sources as a carbon source to produce heterologous proteins, industrial enzymes, and high value-added compounds [16–19].

This study aimed to improve xylonic acid production *by K. phaffii* kXDH-HL strain by comparing the use of glucose and glycerol as carbon sources and applying fed-batch cultures. In addition, refined xylose supplemented with sugarcane bagasse hydrolysate was also evaluated for the production of the acid in bioreactor cultures.

Materials and Methods

All reagents used in this study were research grade and sourced from Sigma-Aldrich.

Strain *K. phaffii* XDH-HL

The recombinant strain *K. phaffii* XDH-HL was employed in this study. For the construction of the strain, the xylB gene from *Halomonas lutea* was cloned under the control of the constitutive promoter pGAP (glyceraldehyde dehydrogenase) and expressed in *K. phaffii* using the integrative vector pGAPZB. Transformants were selected using the antibiotic zeocin, and PCR confirmed the integration of the vector in the yeast genome [7, 15]. The

yeast was preserved in glycerol (30%) at −80 °C, and reactivated on YPD-agar plates supplemented with zeocin (20 $g.L^{-1}$ bacteriological peptone, 20 $g.L^{-1}$ glucose, 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ agar, zeocin 100 μg/mL) when necessary.

Effect of Using Glucose and Glycerol as Carbon Source for Cell Growth on Xylonic Acid Production

Buffered Flask Cultures

Flask cultures were conducted using 250-mL Erlenmeyer flasks and buffered at pH 5.5 by adding 0.2 M potassium phthalate buffer. This buffer solution was prepared by adjusting monobasic potassium hydrogen phthalate (KHP) with KOH (85% purity).

Batch Fermentation

The effects of using glucose and glycerol as carbon sources for xylonic acid production were investigated using batch cultures. Flask cultures were run in 250-mL Erlenmeyer flasks at a working volume of 1:5 v/v, incubated at 30 °C, agitated at 200 rpm, and periodically sampled for 96 h in a shaker/incubator (MAXQ5000, Thermo Scientific). The media used were YP supplemented with 40 $g.L^{-1}$ xylose and either 10 $g.L^{-1}$ glucose or glycerol.

The XDH-HL strain was plated on YPD or YPG plates (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ bacteriological peptone, 20 $g.L^{-1}$ glucose or glycerol, and 20 $g.L^{-1}$ agar). The plates were incubated at 30 °C (Quimis, Q315M25) for 2 days. Plating was done using the depletion technique.

The recombinant strain XDH-HL was inoculated in two stages in YPD or YPG liquid cultures. A test tube culture (10 mL medium in a 50-mL Falcon tube) was inoculated with an isolated colony. The tubes were incubated in a shaker orbital (MAXQ5000, Thermo Scientific) at 200 rpm at 30 °C. After 48 h, the culture was transferred to a 1-L Duran-baffled Erlenmeyer flask containing 250 mL YPD/YPG and grown under the same conditions for 24 h. Cells were harvested by centrifuging for 10 min at 2717 × rcf using a bench centrifuge (Thermo Scientific). The yeast cells were resuspended in dH_2O. Fermentations were initiated at an optical density (OD_{600}) of 5.

Regular and Fed-Batch Flask Cultures

Batch cultures with and without pulse feeding of glucose/glycerol were grown in 250-mL Duran-baffled Erlenmeyer flasks, at 30 °C, 200 rpm for 190 h in an orbital shaker (Mutitron Standard, Infors). The medium was either YPD or YPG, and cultures were inoculated to an OD_{600} of 10. Cultures were periodically sampled for OD_{600}, glucose, glycerol, xylose, and xylonic acid.

Xylose was exhausted in batch cultures within 24 h. Therefore, xylose was manually added to fed-batch cultures every 24 h from a sterile concentrated xylose solution (200 $g.L^{-1}$). Enough xylose stock was added at 24, 48, and 72 h to bring the culture to a concentration of 20 $g.L^{-1}$. After 96 h, the concentration was brought to 40 $g.L^{-1}$ and at 144 h to 30 $g.L^{-1}$.

Glucose or glycerol was fed separately from xylose. At 96 h, the glucose or glycerol concentration was replenished to 20 g. L^{-1} using a 200 $g.L^{-1}$ sterile stock solution.

Xylonic Acid Production in a Bioreactor Controlled at Constant pH and Aerotion

Fed-batch cultures were run with a starting volume of 400 mL of YPG with 20 $g.L^{-1}$ xylose using a 1 L bioreactor (Eppendorf, Dasgip Bioblock), equipped with a Rushton impeller. Bioreactors were controlled at 30 °C and pH 5.5, stirred at 200 rpm, and aerated with air at 0.8 $L.min^{-1}$. Every 24 h from 24 to 216 h, xylose was added (from a 200 $g.L^{-1}$ stock) to bring its concentration to 20 $g.L^{-1}$. Glycerol was added at 48 h to a final concentration of 20 $g.L^{-1}$ from a 200 $g.L^{-1}$ glycerol stock. Fermentations were sampled every 24 h and ended at 240 h.

Xylonic Acid Production in Fermentations with Sugarcane Bagasse Hydrolysate

Mineral Medium for Fermentation with Sugarcane Bagasse Hydrolysate

For the fermentations with sugarcane bagasse hydrolysate (10, 20, and 30%), the mineral medium was composed of 25% ($v.v^{-1}$) of salts solution (50 $g.L^{-1}$ ammonium sulfate, 5 $g.L^{-1}$ magnesium sulfate, and 30 $g.L^{-1}$ monobasic potassium phosphate), 0.25% ($v.v^{-1}$) vitamin solution, 0.25% ($v.v^{-1}$) trace element solution, and 4 $g.L^{-1}$ urea solution. The medium was supplemented with 200 $g.L^{-1}$ xylose stock solution and 200 $g.L^{-1}$ glycerol, resulting in an initial xylose concentration of 40 $g.L^{-1}$ and a glycerol concentration of 10 $g.L^{-1}$. The vitamin and trace element solutions were prepared in accordance with the methodology outlined by Verduyn et al. [20].

Sugarcane Hydrolysate Batch Fermentations

Fermentations were carried out with a hydrolysate that was diluted in the cultivation medium to final concentrations of 10, 20, and 30% ($v.v^{-1}$). For this, a 1-L Bioreactor (Infors HT) was used, containing 700 mL of minimum medium, which included a salt solution (25% $v.v^{-1}$), trace element solution (0.25% $v.v^{-1}$), vitamin solution (0.25% $v.v^{-1}$), and urea 4 $g.L^{-1}$ solution, supplemented with the hydrolysate and 40 $g.L^{-1}$ xylose and 10 $g.L^{-1}$ glycerol. Fermentation took place at 30 °C, 400 rpm stirring speed, and a steady state compressed air flow of 0.8 $L.min^{-1}$. The pH was kept constant at 5.5 by the online addition of 3 M KOH. The fermentation lasted 96 h, and aliquots were withdrawn every 24 h.

The sugarcane bagasse hydrolysate that was utilized in the fermentation processes was produced via steam explosion and acid hydrolysis of the hemicellulose-rich fraction [21, 22].

Before use, the hydrolysate was sterilized by filtration. For this, particles were removed by centrifugation at a Bench Centrifuge from Thermo Scientific, stirring at 4700 rpm for 10 min. Then, pH was adjusted to 5.5 with 85% ($v.v^{-1}$) KOH solution, centrifuged again under the same conditions, and finally filtered using a vacuum pump and a 0.22 µm membrane from the Milipore Filtration System. The filtration process was conducted in a sterile medium using a Laminar Flow Hood from the Filter Flux brand.

The sugarcane bagasse hydrolysate contained 5.81 ± 0.08 $g.L^{-1}$ glucose, 91.15 ± 1.77 $g.L^{-1}$ xylose, and 21.47 ± 0.46 $g.L^{-1}$ acetic acid.

Analytical Methods

Optical density (OD) of the XDH-HL *K. phaffii* strain was measured with appropriate dilution using a UV–visible spectroscopy system at 600 nm (Implen). Dry cell weight determination was done by drying cells at 70 °C overnight to constant weight. After collecting a few data points and using linear regression, we were able to obtain the constant for biomass ($g{\cdot}L^{-1}$) by Eq. 1.

$$Biomass = 2.175 \cdot OD - 0.128 \tag{1}$$

Cell viability was assessed using the Neubauer Camera Yeast/Spore Quantification method, which counts living and dead yeast cells per unit volume of a suspension in a microscope with a 40× objective (BEL Photonic Trinocular Microscope). A solution with erythrosine (0.196 g. L^{-1}) at phosphate buffered 0.1 M was used to dye dead cells. Cell counting was performed in triplicate, and the percentage of living cells was calculated as the average of the results. Equation 2 was used to evaluate cell viability as follows:

$$Cellviability(\%) = \frac{Totalviablecells(living)}{Totalcells(viable + notviable)}\ 100 \tag{2}$$

Quantification of substrates (xylose, glycerol, and glucose) and fermentation product (xylonic acid) was carried out using Ultra High Pressure Liquid Chromatography (UHPLC) on the Waters UPLC Acquity H-Class equipment equipped with an ELSD detector and BEH Amida Premium chromatographic column (2.1 mm × 100 mm; 1.7 µm). Two mobile phases were used: the first solution was composed of acetonitrile/water 80:20, 10 mM ammonium acetate, 0.2% ($v.v^{-1}$) ammonium hydroxide, and distilled water purified in a Milli-Q system; the second solution was composed of acetonitrile/water 40:60, 10 $mmol.L^{-1}$ ammonium acetate, 0.2% ($v.v^{-1}$) ammonium hydroxide, and distilled water purified in a Milli-Q system. The mobile phase flow rate was 0.3 $mL.min^{-1}$. Before UHPLC analysis, samples collected during fermentations were immediately centrifuged at 14,000 rpm for 15 min to remove cells, and the supernatants were stored at −20 °C until analysis.

The concentrations of acetic acid, xylose, and glucose present in the sugarcane bagasse hydrolysate were determined by a high-performance liquid chromatography (HPLC) system (Agilent, 1260 infinity). Column 300×7.8 mm Aminex HPX-87H column; precolumn (Bio-Rad) 30×4.6 mm; mobile phase 0.005 $mol{\cdot}L^{-1}$ H_2SO_4; flow rate 0.6 $mL.min^{-1}$; RID detector 40 °C and column temperature 45 °C.

Fermentation Yield

The bioconversion of substrate into the product ($g_{product}{\cdot}g_{substrate}{}^{-1}$) was calculated by Eq. 3 to determine the average fermentation yield, with xylonic acid as the product and consumed xylose as the substrate. The average volumetric productivity ($g{\cdot}L^{-1}{\cdot}h^{-1}$) was determined according to Eq. 4. The average substrate consumption rate was determined by the amount of xylose consumed in relation to the fermentation time ($g_{substrate}{\cdot}L^{-1}{\cdot}h^{-1}$), as shown in Eq. 5.

$$\overline{\gamma}_{\frac{P}{S}} = -\left[\frac{\left(\frac{dP}{dS}\right)_a + \left(\frac{dP}{dS}\right)_b + \left(\frac{dP}{dS}\right)_c}{n}\right] = -\left[\frac{\left(\frac{P_f - P_i}{S_f - S_i}\right)_a + \left(\frac{P_f - P_i}{S_f - S_i}\right)_b + \left(\frac{P_f - P_i}{S_f - S_i}\right)_c}{n}\right] \tag{3}$$

$$\bar{r}_P = -\left[\frac{\left(\frac{dP}{dt}\right)_a + \left(\frac{dP}{dt}\right)_b + \left(\frac{dP}{dt}\right)_c}{n}\right] = -\left[\frac{\left(\frac{P_f - P_i}{t_f - t_i}\right)_a + \left(\frac{P_f - P_i}{t_f - t_i}\right)_b + \left(\frac{P_f - P_i}{t_f - t_i}\right)_c}{n}\right] \quad (4)$$

$$\overline{V} = \left[\frac{\left(\frac{dS}{dt}\right)_a + \left(\frac{dS}{dt}\right)_b + \left(\frac{dS}{dt}\right)_c}{n}\right] = \left[\frac{\left(\frac{S_f - S_i}{t_f - t_i}\right)_a + \left(\frac{S_f - S_i}{t_f - t_i}\right)_b + \left(\frac{S_f - S_i}{t_f - t_i}\right)_c}{n}\right] \quad (5)$$

where $\overline{\gamma}_{P/S}$ represents the average conversion factor, $\bar{r}_P$ is the average productivity rate, and P is the product concentration ($g{\cdot}L^{-1}$) given as the difference between the final (P_f) and initial (P_i) product concentrations. S is the substrate concentration ($g{\cdot}L^{-1}$) given as the difference between the final (S_f) and initial (S_i) concentrations of the substrate. $\overline{V}$ is the average substrate consumption rate ($g{\cdot}L^{-1}{\cdot}h^{-1}$), and t is time (h). The experiments were performed in triplicate; thus, a, b and c represent each replicate, and n corresponds to the total number of replicates.

The Monod model measures biomass (X) as the dry weight of cells per unit volume. The instantaneous cell growth rate μ (h^{-1}) represents the growth rate at a specific moment, that is, the derivative of cell concentration in relation to time (Eq. 6). The mean cell growth rate ($\overline{\mu}$) represents the average speed of cell growth in a finite interval of time (Eq. 7). The rate of cell mass formation, R_X ($g{\cdot}L^{-1}{\cdot}h^{-1}$), and the maximum specific growth rate (μ_{max}) were calculated by plotting the graph Ln $X{\cdot}X_o^{-1}$ as a function of time. The angular coefficient of the straight line adjusted to the experimental data during the exponential phase of growth corresponds to μ_{max} [23].

$$\mu_X = \frac{1}{X}.\frac{dX}{dt} \quad (6)$$

$$\overline{\mu} = \frac{ln(X_2) - \ln(X_1)}{t_2 - t_1} \quad (7)$$

$$R_X = \mu X_0 \quad (8)$$

$$X = X_0 e^{\mu_{max}(t - t_0)} \quad (9)$$

Results and Discussion

XylonicAcid Production When Using Glucose or Glycerol as Carbon Sources for Cell Growth

K. phaffii strain XDH-HL has been engineered to oxidize xylose to xylonic acid by expressing the xylose dehydrogenase *Halomonas lutea*, as described in an earlier study. Regular and fed-batch fermentations were carried out to evaluate the effect of using glucose and glycerol as carbon sources for the growth of this yeast and on the production of xylonic

acid from xylose. Glycerol is of particular interest because it is a byproduct of biodiesel production. Thus, incorporating glycerol into other bioprocesses not only enhances the economics of the biodiesel production chain but also contributes to sustainability [10].

The yeast grew to a higher cell density on glycerol than on glucose by about 16% (Fig. 1B). The greater growth is likely because glycerol was metabolized throughout the fermentation, while glucose was exhausted within 6 h. Understanding the growth characteristics of the microorganisms utilized in bioprocessing is critical since the desired result may be dependent on cell development [24].

The choice of using glucose or glycerol as the carbon source affected both sugar consumption and xylonic acid yield. While not all of the xylose was consumed in the glycerol culture, the final xylonic acid concentration was higher than for the glucose culture (Fig. 1). The added glucose (12.35 ± 0.31 g.L^{-1}) was exhausted within 6 h, and the final yield of xylonic acid was 20.30 ± 0.31 g.L^{-1} (Fig. 1A). In contrast, when growing on glycerol (beginning with 12.99 ± 0.35 g.L^{-1}), glycerol was exhausted within 96 h, and the final xylonic yield was 24.88 ± 1.64 g.L^{-1} (Fig. 1B).

Glycerol is superior to glucose as a carbon source for producing xylonic acid (Table 1). The glycerol culture produced 18.4% more xylonic acid and had a 9% higher yield and 19% higher productivity compared to that with glucose. Interestingly, xylose was exhausted in the glucose but not in the glycerol culture, even after 96 h.

Glycerol has been confirmed as an effective carbon source for the growth of the recombinant *K. phaffii* strain XDH-HL. This is due to the presence of four genes in the genome that code for H+/glycerol symport proteins, as previously predicted in earlier studies [17]. Glycerol represents an attractive carbon source for bioprocesses due to its low cost and high abundance as a biodiesel byproduct. Its utilization as a fermentation substrate adds value to the biodiesel production chain while providing an economical feedstock for microbial growth [16].

Based on the batch fermentation results, cell growth occurred during the initial phase, supported by glucose or glycerol as carbon sources. The conversion of xylose to xylonic acid increases after 24 h of fermentation. To favor cell growth, approximately 40 g.L^{-1} of glucose or glycerol was initially added. Subsequently, xylose was supplemented every 24 h to promote xylonic acid production.

In glucose-fed fermentation (Fig. 2A), the process started with 17.59 ± 0.74 g·L^{-1} of xylose and 37.11 ± 1.47 g·L^{-1} of glucose. Glucose was rapidly consumed and fully depleted within 24 h, consistent with previous batch results (Fig. 1A). An additional 20 g·L^{-1} of glucose was supplied at 96 h, resulting in a residual concentration of 15.93 ± 0.29 g·L^{-1} at 118 h, and full consumption by 166 h. The process lasted 190 h and produced 20.91 ± 1.05 g·L^{-1} of xylonic acid.

In the glycerol-fed process (Fig. 2B), initial concentrations were 21.06 ± 1.48 g·L^{-1} of xylose and 41.43 ± 1.53 g·L^{-1} of glycerol. Glycerol depletion occurred more slowly, with 25.63 ± 2.74 g·L^{-1} remaining at 22 h and full consumption around 72 h. A second pulse of 20 g·L^{-1} glycerol was added at 96 h, decreasing to 12.25 ± 2.71 g·L^{-1} by 118 h and being fully consumed by 166 h.

The xylose concentration profile decreased only in the first 24 h (Fig. 2 B). To maintain levels above 20 g.L^{-1}, a 200 g.L^{-1} xylose stock solution was added every 24 h. After 190 h of fermentation, the medium still contained 47.01 ± 0.24 g.L^{-1} of xylose, and 20.87 ± 0.30 g.L^{-1} of xylonic acid was produced.

As shown in Fig. 2, the results indicate that cell growth was higher in pulse batch fermentations when glycerol was used as the carbon source, as well as in the batch fermentation. Despite identical initial biomass concentration (21.63 g.L^{-1}), cell density increased

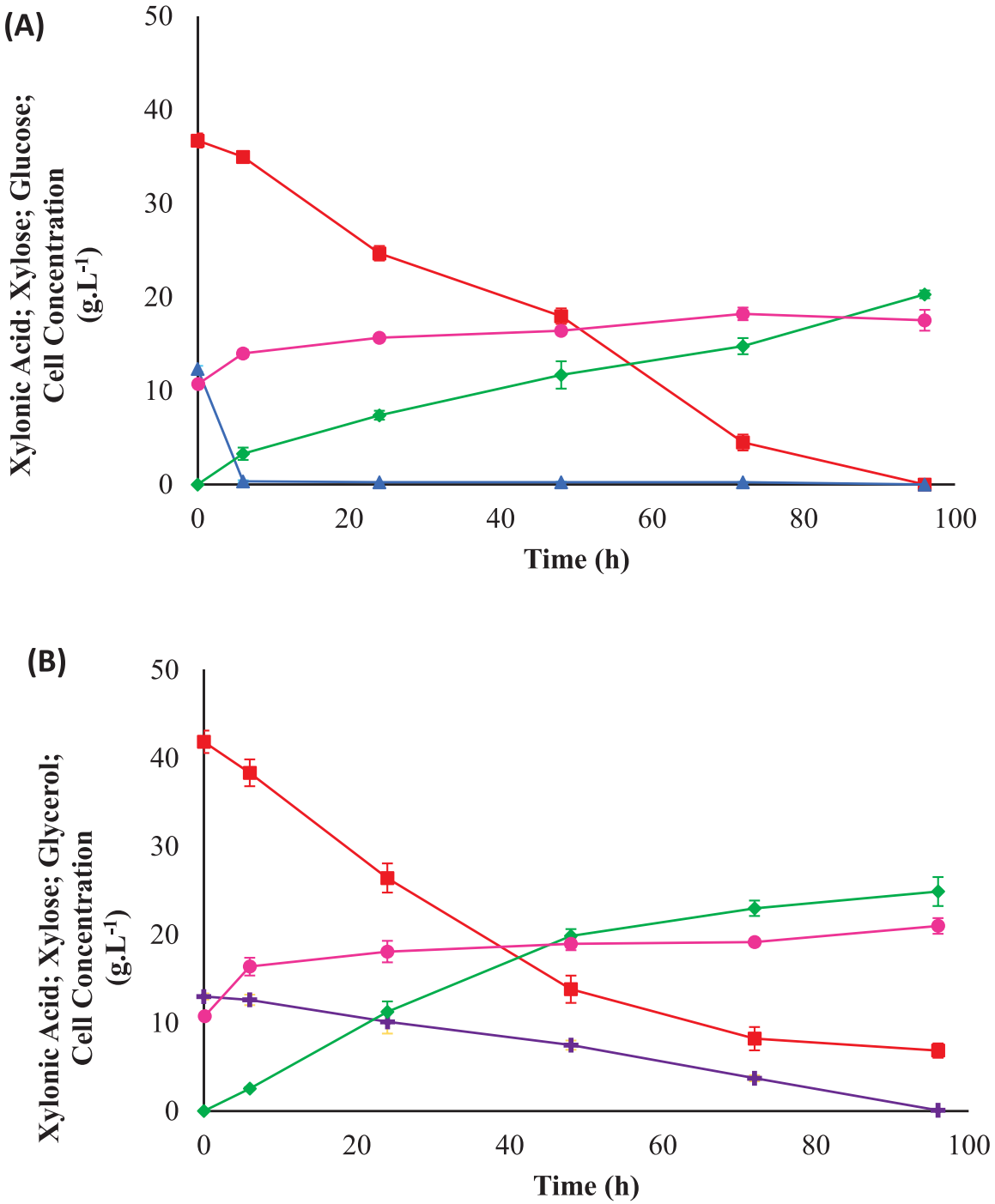

Fig. 1 Flask batch culture with glucose/xylose (**A**) and glycerol/xylose (**B**): xylose (square, ■), glucose (triangle, ▲), glycerol (+), xylonic acid (diamond, ◆), and cell concentration (circle, ●) during fermentations were carried out with $Y_{P/S}$ supplemented with xylose 40 g.L^{-1} and glucose 10 g.L^{-1} (**A**) or xylose 40 g.L^{-1} and glycerol 10 g.L.$^{-1}$ (**B**)

throughout the fermentation in glycerol-fed cultures (Fig. 2 B), indicating that glycerol supported sustained biomass formation.

As the media were fed during fermentation, the substrate's conversion factor to product $Y_{P/S}$ ($g_{xylonic\ acid}.g_{xylose}^{-1}$), productivity ($g_{xylose\ acid}.L^{-1}.h^{-1}$), and xylose substrate consumption rate ($g_{xylose}.L^{-1}.h^{-1}$) were calculated based on the total xylose added to the medium and the xylonic acid produced, as shown in Table 2.

Table 1 Parameters related to batch fermentations with glucose and fermentations with glycerol

Parameters	Co-substrate Glucose	Co-substrate Glycerol
Xylonic Acid produced ($g.L^{-1}$)	20.30 ± 0.41	24.88 ± 1.64
YP/S ($g_{xylonic\ acid}\cdot g_{xylolse}^{-1}$)	0.50 ± 0.01	0.55 ± 0.03
Productivity ($g_{xylonic\ acid}.L^{-1}.h^{-1}$)	0.21 ± 0.00	0.26 ± 0.02
Xylose consumption rate ($g_{xylose}.L^{-1}.h^{-1}$)	0.38 ± 0.01	0.36 ± 0.01
Residual Xylose ($g.L^{-1}$)	0.00	6.86 ± 0.81

The initial substrate concentrations are presented in Fig. 1.

After 190 h, fermentations with glucose and glycerol yielded similar xylonic acid concentrations, substrate consumption rates, and productivities. However, glucose fermentations resulted in a higher substrate-to-product conversion ($Y_{P/S}$) of 0.46 ± 0.03 $g_{xylonic\ acid}\cdot g_{xylose}^{-1}$ compared to glycerol fermentations with 0.37 ± 0.01 $g_{xylonic\ acid}\cdot g_{xylose}^{-1}$, indicating more efficient xylose conversion. In pulse-batch fermentations, both yield and productivity were lower than in batch fermentations, likely due to a gradual decline in the yeast's conversion efficiency over time.

These are fermentations in an Erlenmeyer flask, so the volume of fermentation medium against the total volume of the Erlenmeyer flask affects the process. In a batch with pulse fermentation, the volume of the Erlenmeyer flask increases, but the amount of accessible oxygen decreases. During batch fermentations, 20% of the total volume of the Erlenmeyer flask was used. The final volume occupied after all feedings was 82 mL, which accounted for 32.8% of the Erlenmeyer flask's capacity. These findings suggest that oxygen limitation likely occurred in flask fermentations, as indicated by the observed growth dynamics.

Aerobic fermentation generates reactive oxygen species, which can induce oxidative stress in microbial cells. Although yeast possesses enzymatic and non-enzymatic defense systems to mitigate oxidative damage [25], excessive reactive oxygen species accumulation, particularly under limited oxygen conditions, may overwhelm these defenses, impairing cell growth and substrate conversion efficiency [26].

Based on the results of batch and batch with pulse fermentations, glycerol proved to be an effective carbon source for cell growth in the recombinant strain XDH-HL. Despite differences in initial biomass (10.75 $g.L^{-1}$ in batch fermentations and 21.63 $g.L^{-1}$ in batch with pulse fermentations), both conditions showed enhanced cell growth with glycerol. Additionally, batch fermentations with glycerol had greater xylonic acid concentrations, substrate-to-product conversion factors, and productivity (Table 1).

The significant surplus of glycerol generated as a byproduct of biodiesel production establishes it as a more economical carbon source compared to glucose, which remains constrained by competition with food and feed applications [16, 27]. Beyond its cost advantage and greater availability, the utilization of glycerol in bioprocesses provides added value to the biodiesel production chain through waste stream valorization. Experimental validation demonstrates glycerol's effectiveness as a carbon source for XDH-HL yeast growth in both batch and fed-batch fermentation systems, further supporting its selection as the primary substrate for subsequent microbial cultivation in this study.

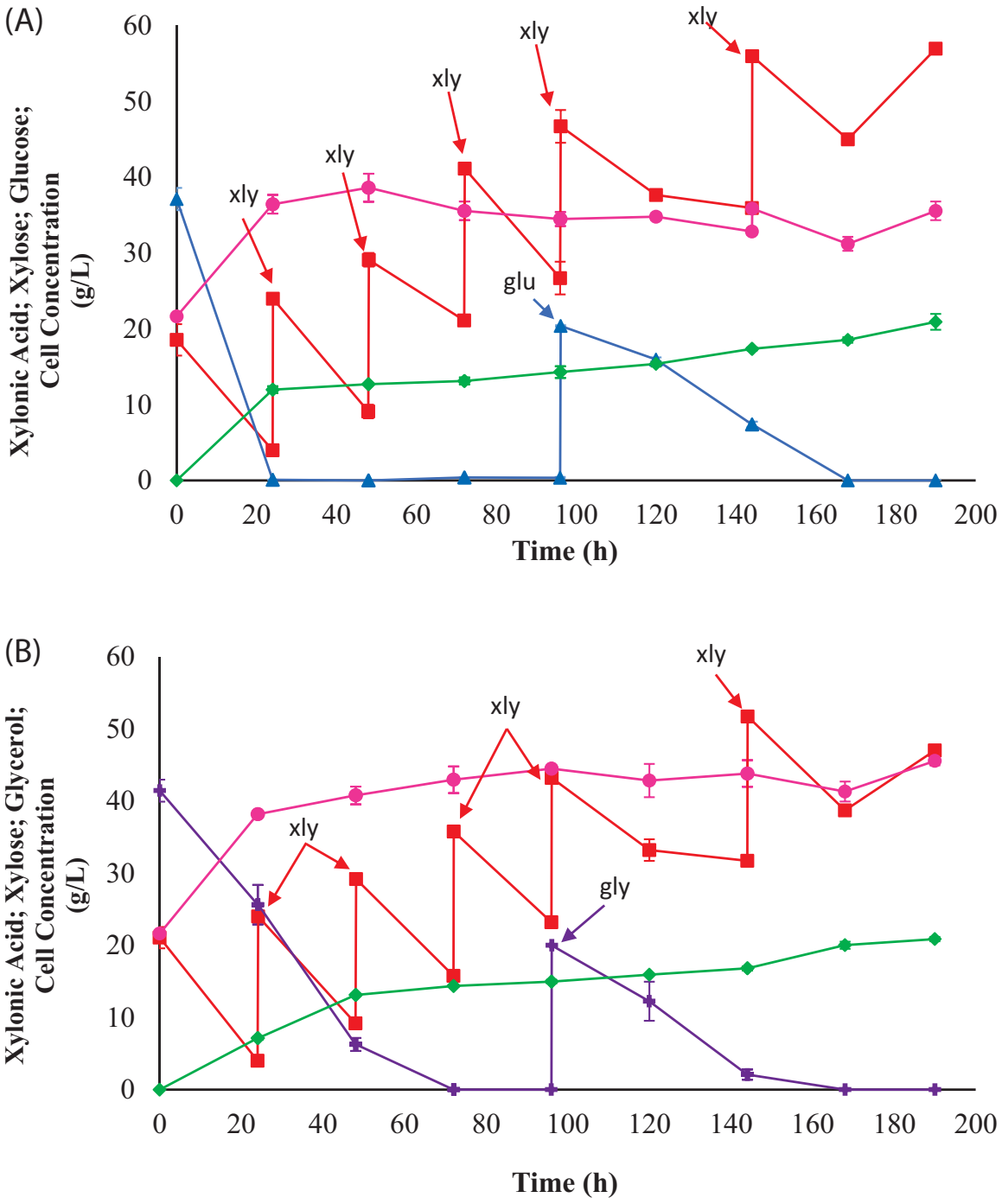

Fig. 2 Flask pulse-batch culture with glucose/xylose (**A**) and glycerol/xylose (**B**): xylose (xly) (square, ■), glucose (glu) (triangle, ▲) glycerol (gly) (+), xylonic acid (diamond, ◆), and cell concentration (circle, ●) during batch with pulse fermentations were carried out with YP supplemented with glucose (**A**) or glycerol (**B**)

Xylonic Acid Production in a Bioreactor with pH Control and Constant Flowrate of Compressed Air

Fermentations were conducted in a bioreactor to precisely monitor/control parameters and increase the final concentration of xylonic acid, enhancing process productivity. A pulse batch cultivation system was used, maintaining constant pH at 5.5 and compressed air flow rate at 0.8 L.min^{-1} throughout the process.

Table 2 Parameters obtained after batch with pulse fermentations with glucose and fermentations with glycerol

Parameters	Co-substrate Glucose	Co-substrate Glycerol
Xylonic Acid produced ($g.L^{-1}$)	20.91 ± 1.05	20.87 ± 0.30
YP/S ($g_{xylonic\ acid}.\ g_{xylolse}{}^{-1}$)	0.46 ± 0.03	0.37 ± 0.01
Productivity ($g_{xylonic\ acid}.L^{-1}.h^{-1}$)	0.11 ± 0.00	0.11 ± 0.00
Substrate consumption rate ($g_{xylose}.L^{-1}.h^{-1}$)	0,04 ± 0.00	0.04 ± 0.00
Residual Xylose ($g.L^{-1}$)	56,91 ± 0.11	47.01 ± 0.24

The initial substrate concentrations are presented in Fig. 1.

A total of 8.49 g of xylose was utilized in each pulse fermentation.

The fermentation started with 19.18 ± 1.51 $g.L^{-1}$ of xylose and 36.71 ± 1.91 $g.L^{-1}$ of glycerol, and after 240 h, 55.11 ± 2.36 $g.L^{-1}$ of xylonic acid was produced (Fig. 3). The xylose concentration decreased only during the first 24 h. Afterward, xylose was added in pulses every 24 h from 24 to 216 h, reaching a final concentration of 20 $g.L^{-1}$, using the required volume of a 200 $g.L^{-1}$ xylose stock solution. Glycerol was added once at 48 h to a final concentration of 20 $g.L^{-1}$, using the necessary volume of a 200 $g.L^{-1}$ glycerol stock solution.

Xylose concentration increased through the bioreactor run (Fig. 3) because the rate of xylose added (every 24 h) exceeded the rate of xylonic acid production. At the end of the fermentation (240 h), 55.11 ± 2.36 $g.L^{-1}$.was produced. During the course of the fermentation, cell density increased > fivefold from 10.75 $g.L^{-1}$ (at 0 h) to 57.08 ± 5.23 $g.L^{-1}$. However, cell viability declined from 100 to 55.41 ± 1.41%.

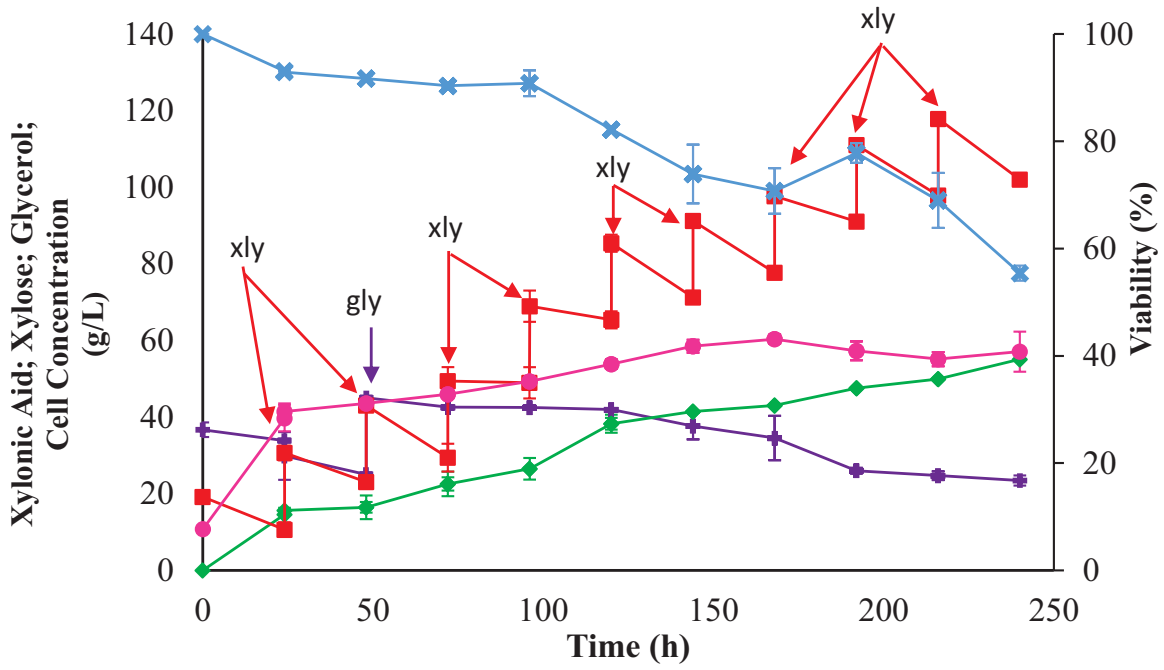

Fig. 3 Bioreactor pulse-batch cultures: xylose (xly) (square, ■), glycerol (gly) (+), xylonic acid (diamond, ◆), cell concentration (circle, •), and viability of XDH-HL yeast (letter x) during batch with pulse fermentation

When comparing the results of pulse batch fermentation in a bioreactor (Table 3) to those in an Erlenmeyer flask (Table 2), all parameters showed better performance in the bioreactor fermentations. The pH in the bioreactor was controlled and maintained at 5.5 throughout the fermentation process. In contrast, during fermentations in an Erlenmeyer flask, pH control was not possible; the medium was only buffered with potassium phthalate at pH 5.5. Furthermore, the amount of oxygen available in Erlenmeyer flask fermentations reduced when more xylose was added to the medium. In contrast, the compressed air flow in bioreactor fermentations was constant at 0.8 L min^{-1}, promoting yeast growth. In addition, the supplementation of compressed air at a flow rate of 0.8 L min^{-1} allowed the DO levels to be maintained above 20% once glycerol was consumed.

Xylonic acid production begins with the enzymatic oxidation of xylose to xylonolactone, which is subsequently hydrolyzed to xylonic acid. This reaction releases protons, leading to acidification of the fermentation medium. The resulting low pH environment inhibits the growth of contaminating microorganisms and reduces the need for neutralizing agents, thereby lowering purification costs and enhancing process economics. Adequate oxygenation is crucial for the regeneration of cofactors such as NAD^+, which is required for dehydrogenase activity during xylose oxidation. In addition to maintaining cofactor balance, oxygen supports cellular respiration, promotes microbial growth, and increases xylonic acid productivity. Thus, the combination of an acidic and well-oxygenated medium contributes to higher fermentation efficiency, improved cell viability, and enhanced product yields [6, 10].

In bioreactor fermentations, 55.11 ± 2.36 g.L^{-1} of xylonic acid was obtained, the productivity was 0.19 ± 0.01 $g_{\text{xylonic acid}}$.L^{-1}.h^{-1}, and the xylose consumption rate was 0.46 g_{xylose}.h^{-1} (Table 2). For fermentations in an Erlenmeyer flask, 20.87 ± 0.30 g.L^{-1} of xylonic acid was obtained, the productivity and speed of consumption of the substrate were 0.11 $g_{\text{xylonic acid}}$.L^{-1}.$h^{-1}$and 0.04 g_{xylose}.h^{-1}, respectively (Table 3). It can be concluded that controlling fermentative factors such as pH and oxygen availability helps to increase process productivity and yield of the desired product.

Lima et al. [18] demonstrated that genetically engineered *K. phaffi* yeast can produce lactate from glycerol in oxygen-restricted fermentations. The GLp strain was created by inserting the L-lactate dehydrogenase (LDH) gene and disrupting the pyruvate decarboxylase (PDC) gene. To test the changed strain, batch fermentations were performed under oxygen limitation. In the end, they had a yield of 67% L-lactic acid and 20% arabitol byproduct [18]. In 2020, this research group conducted a thorough examination of the metabolism of previously modified yeast, with the goal of lowering the creation of the arabitol byproduct while increasing lactic acid production. They carried out fermentations in a bioreactor with 50% dissolved oxygen throughout the process and pH control to keep it

Table 3 Parameters related to batch with pulse fermentations in bioreactor

Parameters	Pulse-batch fermentation (bioreactor)
$\mu_{(\text{specific growth rate})}$ (h^{-1})	$0.17 \pm 0{,}01$
Xylonic acid-produced (g.L^{-1})	55.11 ± 2.36
YP/S ($g_{\text{xylonic acid}} \cdot g_{\text{xylolse}-1}$)	0.97 ± 0.05
Productivity ($g_{\text{xylonic acid}}$.L^{-1}.h^{-1})	0.19 ± 0.01
Substrate consumption rate (g_{xylose}.L^{-1}.h^{-1})	0.46 ± 0.00
Total amount of xylose added (g)	110.31 ± 0.60
Xylose consumed (g)	39.53 ± 0.19

at 5. In this recent study, they found that lactic acid synthesis increased by 20% while arabitol production decreased by 50%. So, they demonstrated that greater control of fermentative parameters in a bioreactor results in increased production of the interest product while reducing the generation of co-products [16].

Xylonic Acid Production from Sugarcane Bagasse Hydrolysate in Bioreactors

During the pre-treatment stages and biomass hydrolysis, cellulose and hemicellulose are broken down, releasing not only monomeric sugars but also phenolic chemicals, furaldehydes, and organic acids [22].These chemicals can restrict microbial metabolism by affecting cell membrane damage, reducing intracellular pH, and inhibiting centeral metabolic enzymes, all of which may negatively impact fermentation parameters such as sugar consumption and product synthesis [22, 28].

To evaluate the inhibitory effect of compounds present in the sugarcane bagasse hydrolysate on xylonic acid production, XDH-HL yeast was cultivated and fermentations were conducted using this hydrolysate at concentrations of 10%, 20%, and 30% relative to the volume of the fermentation medium. The hydrolysate contains 21.47 ± 0.46 g.L^{-1} of acetic acid, 91.15 ± 1.77 g.L^{-1} of xylose and 5.81 ± 0.08 g.L^{-1} of glucose, previously quantified by HPLC. Thus, xylose was supplemented in the fermentation medium, so that the initial concentration was around 40 g.L^{-1} for all fermentations. In addition, the fermentations were carried out using glycerol as the main carbon source for cell growth. In this case, the initial concentration of this sugar was expected to be around 10 g.L^{-1}.

Figure 4 exhibits fermentative profiles. Figure 4A shows the amount of xylonic acid produced and Fig. 4B depicts concentrations of xylose and glycerol during the fermentations. The initial concentration of xylose was 42.33 g.L^{-1}, 36.91 g.L^{-1}, and 36.0 g.L^{-1} in the fermentations with 10%, 20% and 30% hydrolysate in the fermentation medium, respectively. Although the fermentation with 10% hydrolysate started with a higher initial concentration of xylose compared to the others, the xylose was depleted by 72 h. In the 20% hydrolysate fermentation, xylose was over by 96 h. Meanwhile, in the 30% hydrolysate fermentation, xylose was not fully consumed, leaving 25.50 g.L^{-1} in the fermentation medium.

The 30% fermentation utilized a substantially higher volume of hydrolysate compared to the 20% and 10% fermentations. As a result, the inhibitory chemicals in the hydrolysate were in higher concentrations. In this study, acetic acid was identified and quantified as the inhibitor. The hydrolysate contained 21.47 ± 0.46 g.L^{-1} of acetic acid; thus, the fermentation with 30% had 4.50 g of acetic acid, the fermentation with 20% had 3.00 g, and the fermentation with 10% of hydrolysate had 1.50 g of microbial inhibitor. In addition to interfering with microbial metabolism, this chemical has the potential to lower sugar consumption [22].

The xylonic acid concentration profiles (Fig. 4A) show that the concentration of this organic acid was higher throughout the fermentation with 10% hydrolysate. After 96 h of fermentation, the production of xylonic acid was 42.30 g.L^{-1}, 18.86 g.L^{-1} and 17.70 g.L^{-1} for fermentations with 10%, 20%, and 30% hydrolysate, respectively.

Fermentation with 30% hydrolysate led to lower conversion of xylose into xylonic acid due to higher levels of inhibitory chemicals, which reduced xylose consumption. Consequently, better substrate consumption, as seen with 10% hydrolysate, results in improved yeast growth and xylonic acid production.

Figure 5 shows the viability and cell concentration patterns of XDH-HL yeast during fermentation. As can be seen, all procedures start with 100% viability and a cell

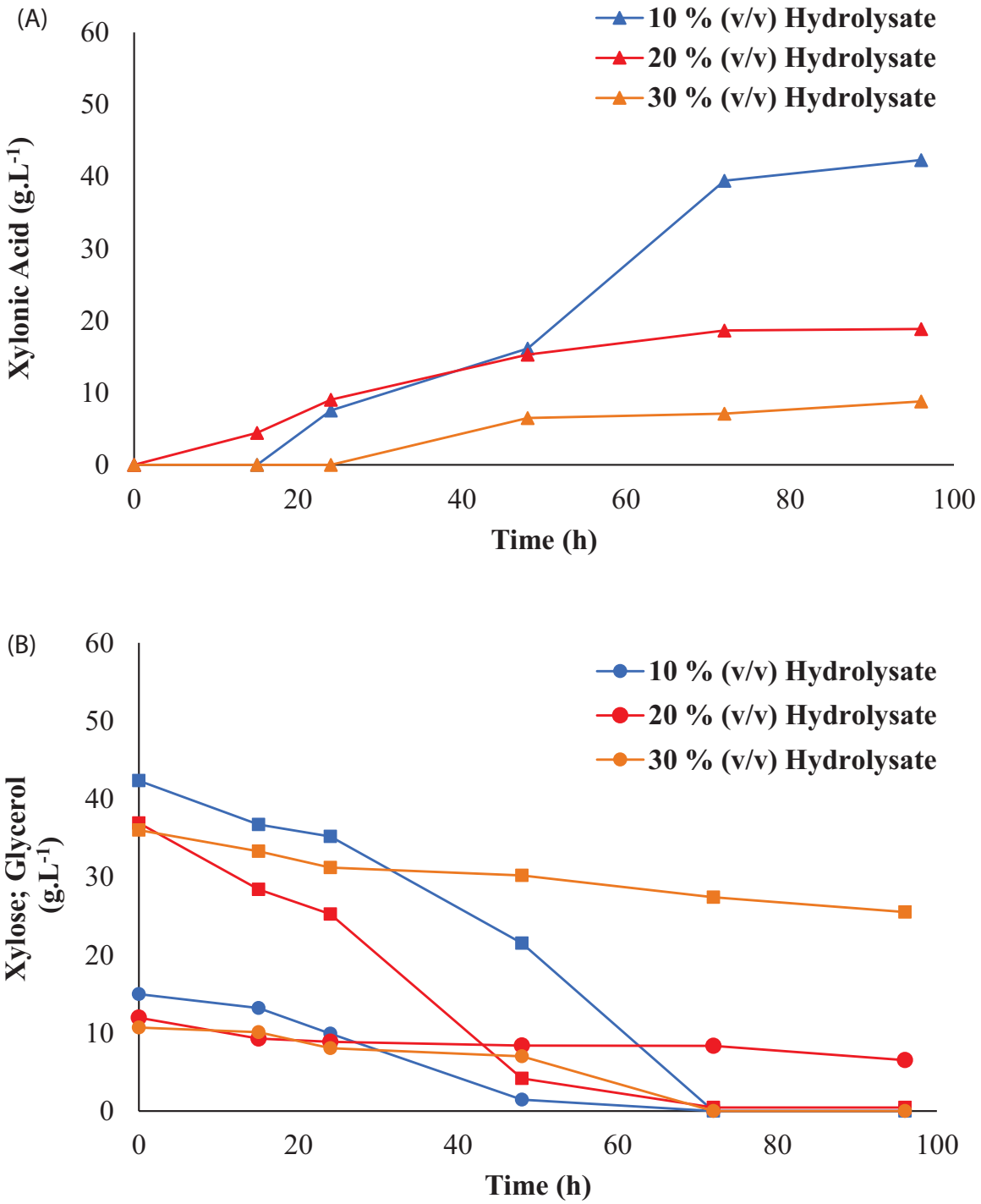

Fig. 4 Fermentative profiles: **A** concentration of xylonic acid (triangle, ▲) and **B** concentrations of xylose (square, ■) and glycerol (circle, ●) during 96 h of fermentations

concentration of 10.75 $g.L^{-1}$. Cell concentration was lower in fermentations with 30% hydrolysate throughout the 96 h.

After 96 h, the fermentation with 30% hydrolysate had the lowest cell viability (81.43%), followed by 20% hydrolysate (82.07%) and 10% hydrolysate (86.78%). Fermentation with 30% hydrolysate resulted in slower cell growth and reduced viability. Furthermore, fermentation variables such as cellular physiological conditions, dissolved oxygen concentration, and pH of the medium also interfere with the toxicity of these compounds, which can accentuate their toxic effect on the microorganism during the xylonic acid production.

Therefore, the presence of toxic compounds, such as acetic acid, can lead to yeast stress and a reduction in the efficiency in the use of sugars, leading to less formation of the product of interest and lower process productivity [29].

After evaluating the concentration profiles of xylose, glycerol, and xylonic acid, the productivity ($g_{xylose}.L^{-1}.h^{-1}$) and xylose substrate consumption rate ($g_{xylose}.L^{-1}.h^{-1}$) were calculated as shown in Table 4.

The specific growth rate (μ) was highest for the fermentation containing 10% hydrolysate (0.17 h^{-1}), followed by the fermentation with 20% (0.16 h^{-1}), and lowest for the fermentation with 30% (0.11 h^{-1}) (Table 4).

According to the results obtained, the fermentation process using 10% hydrolysate had a higher productivity, a higher rate of substrate consumption, and a higher final concentration of xylonic acid. Furthermore, no residual xylose remained in the fermentation medium (Table 4). On the other hand, fermentations with 20% and 30% hydrolysate presented the worst fermentation parameters, with productivity values and final concentration of xylonic acid close to each other. Additionally, the substrate consumption rate was much lower for fermentation with 30% hydrolysate, meaning that at the end of the entire process there was still more than 25 $g.L^{-1}$ of xylose remaining in the fermentation medium. This demonstrates that the amount of hydrolysate employed in fermentations affects the xylonic acid production. When more hydrolysate is added, the final concentration of xylonic acid decreases. This is because the fermentation medium contains a greater concentration of inhibitory chemicals, such as acetic acid. In addition to altering cell growth (Fig. 5), it inhibits xylose consumption (Fig. 4B) and xylonic acid synthesis (Fig. 4A).

In fermentation with 10% v/v hydrolysate, 42.30 $g.L^{-1}$ of xylonic acid was obtained, a value close to that obtained for fermentation in synthetic medium, 55.11 ± 2.36 $g.L^{-1}$ (Table 3). However, productivity was significantly higher with the hydrolysate (0.44 $g.L^{-1}.h^{-1}$) compared to the productivity (0.19 ± 0.01 $g.L^{-1}.h^{-1}$) obtained for fermentation in synthetic medium. Similarly, the yield with 10% hydrolysate reached 1.00 $g_{xylonic\ acid} \cdot g_{xylolse}^{-1}$, exceeding the yield observed in the synthetic medium (0.97 ± 0.05 $g_{xylonic\ acid} \cdot g_{xylolse}^{-1}$).

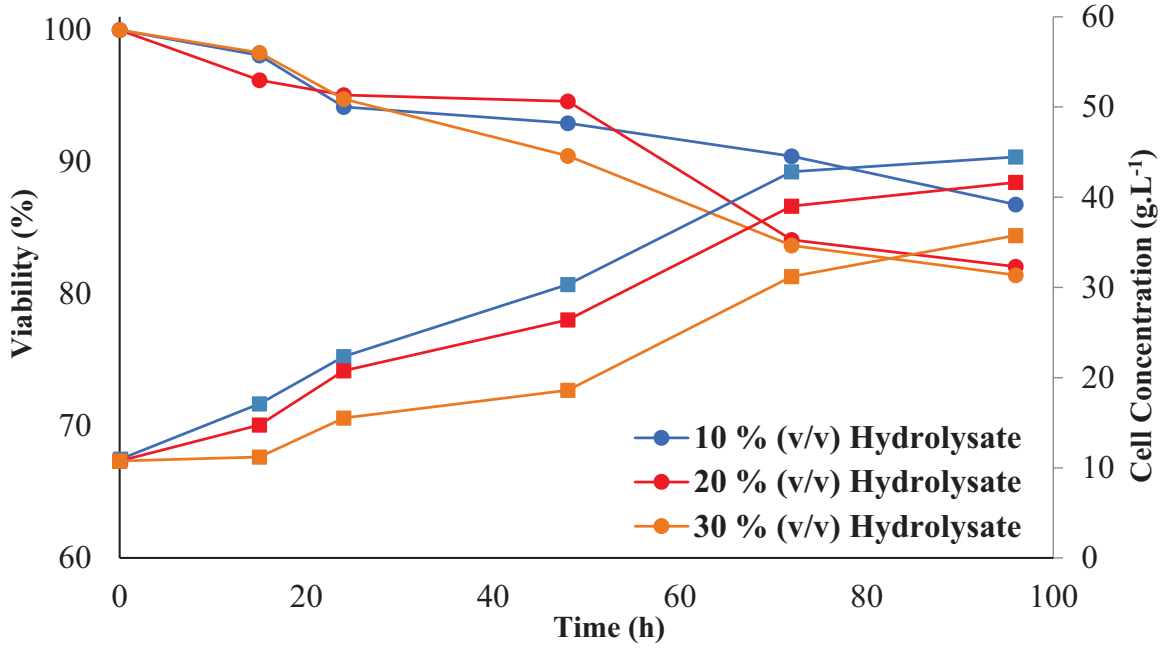

Fig. 5 Viability profiles (circle, •) and cell concentration (square, ■) of XDH-HL yeast during fermentations: 10% (v/v), 20% (v/v) and 30% (v/v) sugarcane bagasse hydrolysate

Table 4 Parameters related to the fermentations carried out with 10% (v/v), 20% (v/v) and 30% (v/v) sugarcane bagasse hydrolysate

Parameters	10 % (v/v) Hydrolysate	20 % (v/v) Hydrolysate	30 % (v/v) Hydrolysate
$\mu_{(\text{specific growth rate})}$ (h^{-1})	0.17	0.16	0.11
Xylonic Acid ($g.L^{-1}$)	42.30	18.85	8.80
YP/S ($g_{\text{xylonic acid}} \cdot g_{\text{xylolse}}{}^{-1}$)	1.0	0.51	0.84
Productivity ($g_{\text{xylonic acid}}.L^{-1}.h^{-1}$)	0.44	0.20	0.10
Substrate consumption rate ($g_{\text{xylose}}.L^{-1}.h^{-1}$)	0.44	0.38	0.11
Initial Xylose(g)	29.63	25.84	25.20
Xylose Consumed (g)	29.63	25.53	7.61

This occurred because in addition to glycerol, the medium contained glucose present in the hydrolysate, which can also be used for cell growth of the yeast. These results demonstrate that the use of 10% sugarcane bagasse hydrolysate is a promising strategy for xylonic acid production with XDH-HL expressing *K. phaffii* strain.

Conclusion

The present study proved that *Komagataella phaffii* XDH-HL effectively produces xylonic acid in a variety of fermentative apparatus. The carbon source used for cell growth and the yeast's fermentative activity were assessed using sugarcane bagasse hydrolysate as a substrate.

Glycerol outperformed glucose as the optimal carbon source for XDH-HL growth in batch and batch with pulse fermentations. Batch fermentations using glycerol achieved a high xylonic acid yield (0.55 ± 0.03 $g_{\text{xylonic acid}} \cdot g_{\text{xylose}}{}^{-1}$), productivity ($0.26 \pm 0.02$ $g_{\text{xylonic acid}}.L^{-1}.h^{-1}$), and titer ($24.88 \pm 1.64$ $g.L^{-1}$). Glycerol enhanced *K. phaffii* XDH-HL growth and fermentation efficiency, outperforming glucose. Therefore, crude glycerol can be proposed as a substrate for cell growth in xylonic acid production, adding value to this biodiesel byproduct. This strategy contributes to both improved xylonic acid yields and greater sustainability of the biodiesel industry.

The batch with the pulse system significantly boosted xylonic acid production, achieving a higher concentration (55.11 ± 2.36 $g.L^{-1}$) and productivity (0.19 ± 0.01 $g_{\text{xylonic acid}} \cdot L^{-1}.h^{-1}$) compared to flask fermentations (20.87 ± 0.30 $g.L^{-1}$, 0.11 $g_{\text{xylonic acid}}.L^{-1}.h^{-1}$). Controlling pH and monitoring oxygen addition in the bioreactor improved substrate utilization, cell viability, and enzymatic efficiency, enhancing yield while reducing contamination risks and operational costs. Optimized conditions can be achieved with oxygen control.

Finally, sugarcane bagasse hydrolysate was added to refined xylose in bioreactor fermentations to assess the production of xylonic acid in the presence of inhibitory substances in the hydrolysate. Adding 10% sugarcane bagasse hydrolysate to commercial xylose boosted xylonic acid production (42.30 $g.L^{-1}$) and yield (0.44 $g_{\text{xylonic acid}} \cdot g_{\text{xylose}}{}^{-1}$). Higher concentrations (20–30%) reduced output (18.85–8.80 $g.L^{-1}$) and productivity (0.20–0.10 $g_{\text{xylonic acid}}.L^{-1}.h^{-1}$), demonstrating inhibitor effects on cell growth and xylose conversion.

Thus, this research showed that the engineered *Komagataella phaffii* strain XDH-HL can efficiently produce xylonic acid using sugarcane bagasse hydrolysate and possibly

employing crude glycerol as a carbon source for cell growth. This approach enhances process sustainability while creating value from industrial waste.

Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Isabela Castro de Almeida. João Ricardo Moreira Almeida, Fabricio Machado, and Sílvia Belém Gonçalves conceived and designed the experiments and analyzed the data. The first draft of the manuscript was written by Isabela Castro de Almeida, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable

Consent to Publish All authors approved the version to be published.

Conflict of Interests The authors declare no competing interests.

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