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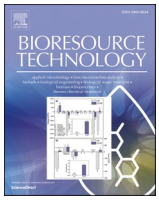

Self-Buffering system for Cost-Effective production of lactic acid from glucose and xylose using Acid-Tolerant *Issatchenkia orientalis*

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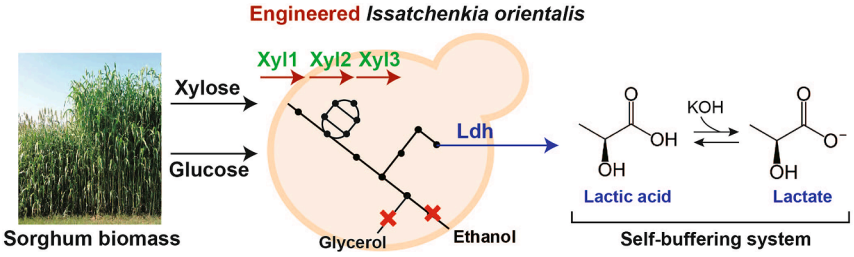
HIGHLIGHTS

- An acid-tolerant yeast, *I. orientalis*, was engineered for organic acid production.
- Engineered *I. orientalis*, SD108XL, can consume xylose and produce lactic acid.
- Without pH regulation, SD108XL had difficulty in producing lactic acid.
- A self-buffering strategy helps produce lactic acid by reducing pH regulation.

GRAPHICAL ABSTRACT



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ABSTRACT

This study presents a cost-effective strategy for producing organic acids from glucose and xylose using the acid-tolerant yeast, *Issatchenkia orientalis*. *I. orientalis* was engineered to produce lactic acid from xylose, and the resulting strain, SD108XL, successfully converted sorghum hydrolysates into lactic acid. In order to enable low-pH fermentation, a self-buffering strategy, where the lactic acid generated by the SD108XL strain during fermentation served as a buffer, was developed. As a result, the SD108 strain produced 67 g/L of lactic acid from 73 g/L of glucose and 40 g/L of xylose, simulating a sugar composition of sorghum biomass hydrolysates. Moreover, techno-economic analysis underscored the efficiency of the self-buffering strategy in streamlining the downstream process, thereby reducing production costs. These results demonstrate the potential of *I. orientalis* as a platform strain for the cost-effective production of organic acids from cellulosic hydrolysates.

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Table 1
Plasmids, strains, and primers used in this study.

Strain	Description	Source
E. coli top 10	Used for cloning	Invitrogen
SD108WT	*Issatchenkia orientalis* wildtype	(Lee et al., 2022)
SD108/ura3Δ	SD108 harboring disrupted *URA3*	(Xiao et al., 2014)
SD108L	SD108/pdcΔ/gpdΔ/ura3Δ/IS1::LDH	This study
SD108X	SD108, xylose pathway integrated into the IS3 region	(Lee et al., 2022)
SD108XL	SD108L, xylose pathway integrated into the IS3 region	This study
Plasmid	Description	Source
pVT36b-PDC	pVT36b with insertion of gBlock for *PDC* deletion	(Tran et al., 2023)
pVT36b-GPD	pVT36b with insertion of gBlock for GPD deletion	(Tran et al., 2023)
pVT36b-int1	pVT36b with insertion of spacer for integration at site 1	(Tran et al., 2023)
pScARS/CEN-L-LDH	Episomal plasmid in *I. orientalis* containing cassette for expression of *LDH* from Leuconostoc mesenteroides	(Cao et al., 2020a)
pCast_IS2	pCast with an IS2 targeting guide RNA expression cassette	(Lee et al., 2022)
pIO-UG_X123	pIO-UG with expression cassettes of P_{TDH3}-*XR*-T_{MDH1}, P_{GPM1}-*XDH*-T_{PDC1}, and P_{TEF1}-*XK*-T_{INO1}	(Lee et al., 2022)
Primer	Sequences (5′-3′)	Source
PDC.check.F	cattgttggaccacgtcaagg	This study
PDC.check.R	aacgagaatgattggtttttctg	This study
GPD.check.F	cccctgctgaaagattatctac	This study
GPD.check.R	aattagcacctgataaggcac	This study
int1.down.R	gaaatgccaagtgtttgagc	This study
LDH.check.F	ttggttacgccacatacg	This study
LDH.F	attccatcccactcctctgaaattaccaaaacatcgatgaaagctcgagttgatttaacc	This study
LDH.R	ttacagctgttaaattatacttgaatttgttttttcggtccgcttggctaaagaataa	This study
F_repair_IS2_X123	aaaaatattgaaccgtcgaaacgtcccaaacaaggaaattgatttaacctgatccaaaagggg	(Lee et al., 2022)
R_repair_IS2_X123	aaggtgagaattcaaaatgttatttttgatcatgtaaaggtatctgtcaacaacgtactggaaaa	(Lee et al., 2022)
F_check_IS2	gcgggcgtgaagtatataagacagacaatatc	(Lee et al., 2022)
R_check_IS2	actaattccagttgagatactcacgcacaaga	(Lee et al., 2022)

1. Introduction

Rapid industrialization based on petrochemical processes has led to significant environmental challenges, such as climate change, due to the emission of flue gases, including CO_2, CH_4, and CO (Delangiz et al., 2019). Thanks to the significant developments in biotechnology, microbial bioprocesses have emerged as a sustainable option for manufacturing various industrial products (Paul et al., 2019; Volk et al., 2023). The Department of Energy (DOE) has identified promising bio-based chemicals, encompassing a variety of organic acids, such as succinic, fumaric, malic, glucaric, aspartic, glutamic, and levulinic acids (Choi et al., 2015). Metabolic engineering of microorganisms facilitates the production of these organic acids, contributing to a more environmentally sustainable production process (Chen & Nielsen, 2016; Tran et al., 2023; Tran & Zhao, 2021). Moreover, the growing interest in biodegradable plastics has further emphasized the importance of bio-based production of monomeric organic acids as building blocks (Liang et al., 2022; Mazzoli, 2020).

The National Renewable Energy Laboratory (NREL) has also underscored the significance of lignocellulosic biorefineries in promoting sustainable industries (Davis et al., 2018). Lignocellulosic biomass, which consists of cellulose, hemicellulose, and lignin, provides abundant carbon sources for microbial fermentation. Notably, hemicellulose and cellulose yield pentose and hexose sugars through the hydrolysis of lignocellulosic biomass (Magalhaes et al., 2019). In order to optimize the use of lignocellulosic biomass in bioprocess, numerous studies have aimed to enable microorganisms to metabolize compounds such as xylose, acetate, and cellobiose (Kwak et al., 2019). Specifically, extensive research on yeast engineering has been conducted for the biological conversion of xylose, the second most abundant sugar, and other carbon sources in lignocellulosic biomass (Kwak et al., 2019). As such, yeast strains have emerged as promising candidates for the industrial production of bioproducts derived from lignocellulosic biomass.

Several studies have reported the bioconversion of lignocellulosic biomass into organic acids using engineered yeasts (Kwak et al., 2019). However, the economic feasibility of the overall bioprocess remains uncertain. During yeast fermentation for organic acid production, which leads to acidic conditions, a neutralization step is necessary to maintain an optimal pH level for yeast growth (Mitsui et al., 2020; Watcharawipas et al., 2021; Xi et al., 2023). This neutralization process dissociates the organic acid into its less soluble salt form, necessitating an additional acidification step to obtain the undissociated acid. This step, however, generates gypsum as a byproduct, which demands further downstream processing for its removal. These downstream processes contribute to increased energy consumption and costs. If low-pH fermentation is feasible, the neutralization step could be eliminated, allowing for the removal of subsequent downstream processes, such as acidification and gypsum purification. This approach is anticipated to reduce energy consumption and the overall cost of microbial organic acid production (Marchesan et al., 2021; Tran et al., 2023). Additionally, low pH fermentation may help minimize the risk of contamination because neutral pH conditions are typically optimal for microorganisms (Xi et al., 2023).

The yeast strain *Issatchenkia orientalis*, also referred to as *Pichia kudriavzevii*, *Candida glycerinogenes*, and *Candida krusei*, exhibits high tolerance to unfavorable conditions for microbial growth (e.g., high temperatures or low pH levels) and inhibitors in lignocellulosic hydrolysates (e.g., furan derivatives) (Douglass et al., 2018; Lee et al., 2022). Indeed, *I. orientalis* showed improved cell growth and ethanol production when subjected to high temperatures, acidic environments, and furfural derivatives as compared to well-known yeast strains such as *Saccharomyces cerevisiae* (Seong et al., 2017). Thus, low-pH fermentation using lignocellulosic hydrolysates appears to be feasible with *I. orientalis*.

In this study, an efficient bioprocess to produce lactic acid from glucose and xylose using *I. orientalis* was developed. Through metabolic engineering, *I. orientalis* was enabled to consume xylose and produce lactic acid. Lactic acid was successfully produced from sorghum hydrolysates containing glucose and xylose. An innovative self-buffering strategy for a low-pH fermentation approach was also devised to reduce the use of neutralizing agents and simplify the downstream process when producing organic acids. As such, a cost-effective bioprocess for organic acid production using glucose and xylose was successfully developed with *I. orientalis*.

2. Materials and methods

2.1. Strain construction

All strains and plasmids used in this study were described in Table 1. *I. orientalis* strains were maintained and grown on YP medium (10 g/L of yeast extract and 20 g/L of peptone) containing 20 g/L of glucose at 30 °C, and 120 μg/L of nourseothricin (cloNAT) was supplemented as a selection marker when required. *Escherichia coli* TOP 10 (Invitrogen, USA) was used for cloning. *E. coli* transformants were grown on Luria-Bertani (LB) medium (5 g/L of yeast extract, 10 g/L of tryptone, and 10 g/L of sodium chloride) at 37 °C with 100 μg/mL ampicillin.

2.2. Yeast strain construction

All transformation methods used in this study were based on the previous lithium acetate/single-stranded carrier DNA/polyethylene glycol method using a CRISPR/Cas9 system with some modifications (Lee et al., 2022). Heat-shock treatment for *I. orientalis* was maintained at 42 °C for 90 min, and the heat-shocked cells were recovered in YP medium for 4 h before plating the cells on YPD agar plates with 120 μg/mL of nourseothricin (cloNAT). In order to construct the Cas9-based genome-edited strains, the pCast plasmid containing Cas9 and guide RNA expression cassettes were used (Lee et al., 2022). For *PDC* deletion, plasmid pVT36b-PDC was transformed into strain SD108/ura3Δ. For verification of deletion, the *PDC* gene was PCR-amplified using primers PDC.check.F and PDC.check.R from genomic DNA, and the PCR product was digested with *Eco*RI. Successful deletion of *PDC* resulted in 2 bands on the agarose gel. Plasmid pVT36b-PDC was then cured using SC-FOA, resulting in strain SD108/pdcΔ/ura3Δ. For *GPD* deletion, plasmid pVT36b-GPD was transformed into strain SD108/pdcΔ/ura3Δ. For verification of deletion, the *GPD* gene was PCR-amplified using primers GPD.check.F and GPD.check.R from genomic DNA, and the PCR product was digested with *Eco*RI. Successful deletion of *GPD* resulted in 2 bands on the agarose gel. Plasmid pVT36b-GPD was then cured using SC-FOA, resulting in strain SD108/pdcΔ/gpdΔ/ura3Δ. For the integration of D-lactate dehydrogenase gene from *Leuconostoc mesenteroides* (*LmLDH*) (Cao et al., 2020b), the LDH cassette was PCR-amplified from plasmid pScARS/CEN-L-LDH using primers LDH.F and LDH.R and co-transformed with pVT36b-int1 into SD108/pdcΔ/gpdΔ/ura3Δ. Yeast colonies were screened for integration of the LDH cassette by PCR using primers LDH.check.F and int1.down.R. Plasmid pVT36b-int1 was then cured using SC-FOA, leading to strain SD108/pdcΔ/gpdΔ/LDH/ura3Δ (SD108L). For introducing xylose-fermenting pathway, the expression cassette including xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulose kinase (XK) from *Scheffersomyces stipitis* was generated by PCR amplification using plasmid pIO-UG_X123 with primers of F_repair_IS2_X123 and R_repair_IS2_X123 (Table 1). The resulting amplicon was used as donor DNA and integrated into the previously identified intergenic sites of chromosome 2 (named IS2) (Lee et al., 2022). The amplified DNA fragments and Cas9-gRNA plasmids (pCas-t_IS2) were co-transformed into the SD108L strain. Correct integration of the XR, XDH, and XK expression cassette in the IS2 region was confirmed by PCR amplification with F_check_IS2 and R_check_IS2 primers.

2.3. Shake-flask fermentations

For shake-flask fermentations, *I. orientalis* strains were cultured in a 125-mL shake-flask with 25 mL of medium. The cultures were performed at 30 °C and 100 rpm for microaerobic conditions. When neutralization was required, 50 g/L of calcium carbonate ($CaCO_3$) was used as a neutralizing agent. Optical density at 600 nm (OD_{600}) was measured using 0.2 M HCl to dilute culture instead of deionized water to prevent interference by residual $CaCO_3$. In shake-flask fermentations, various fermentations were conducted using YP and synthetic complete (SC) medium (6.7 g/L yeast nitrogen base and 0.79 g/L complete supplement mixture (MP Biomedicals, Solon, OH, USA)) containing glucose and xylose, as well as sorghum biomass hydrolysate.

2.4. Bioreactor fermentations

Bioreactor fermentations were conducted in a DASbox mini bioreactor system (Eppendorf, Hamburg, Germany). The culture volume of each bioreactor was 100 mL. Glucose and xylose were added to YP medium to mimic the sugar concentrations of lignocellulosic biomass hydrolysates. The fermentation was performed at 30 °C, 7.5 standard liter (sL)/h of an airflow rate, and 100 rpm of an agitation rate. pH was controlled by using 4 N KOH solutions to investigate the effect of pH on the culture.

2.5. Quantitative analysis

Cell growth was measured by OD_{600} using a UV–visible spectrophotometer (BioMate 3S; Thermo Fisher Scientific, Waltham, MA, USA). Sugars and extracellular metabolites were determined and quantified using high-performance liquid chromatography (HPLC, Agilent 1200 Series; Agilent Technologies, Wilmington, USA) equipped with a refractive index detector and Rezex ROA-Organic Acid H+ (8 %) column (Phenomenex Inc., Torrance, CA, USA). The samples were analyzed at 50 °C with 0.005 M H_2SO_4 as the mobile phase, with a flow rate of 0.6 mL/min. The culture broth was centrifuged at 13,000 rpm for 10 min, and supernatants were obtained to analyze extracellular metabolites and sugars in the medium.

2.6. Techno-economic analysis

Superpro Designer v9.5 (Intelligen, Inc., Scotch Plains, NJ, USA) was used to calculate the mass and energy balances of the processes. The following units of the process (see supplementary material) were modeled in Superpro Designer: four blending tanks for making medium (three) and gypsum washing (one), four 400 tons fermentors for lactic acid production, three pasteurizer for medium sterilization, two neutralizers for lactate acidification, two hydrocylones for gypsum removal, seven flat bottom tanks for product storage in the middle of the process (see supplementary material).

Capital costs were derived from direct and indirect expenses, using Superpro Designer v9.5 values. The annualized capital cost is calculated by multiplying the total capital cost by the capital recovery factor (CRF), which can be calculated as follows:

$$\mathrm{CRF} = \frac{i(1+i)^N}{(1+i)^N - 1} \tag{1}$$

where 'i' is the interest rate (8 %) and 'N' is the plant's operational lifespan (20 years), following assumptions from previous studies (Heo et al., 2019).

The annual operating cost was calculated as the sum of materials, utilities, labor, maintenance, and Laboratory QC/QA costs. The material and utility costs were estimated based on the simulation results and unit prices of the chemicals and utilities. The labor cost is estimated using the following correlation equation (Heo et al., 2019):

$$\text{Labor cost} = 10^6 \times \left(\frac{\text{Total capital cost}}{10^6 \times 500}\right)^{0.2} \tag{2}$$

The maintenance cost was estimated to be 2 % of the total capital cost, and the laboratory cost for quality control and assurance (Laboratory QC/QA) was estimated to be 8 % of the labor cost (Kwan et al., 2015). Finally, the annual cost was defined as the sum of the annualized capital cost and the annual operating cost. The lactic acid production cost was defined as follows:

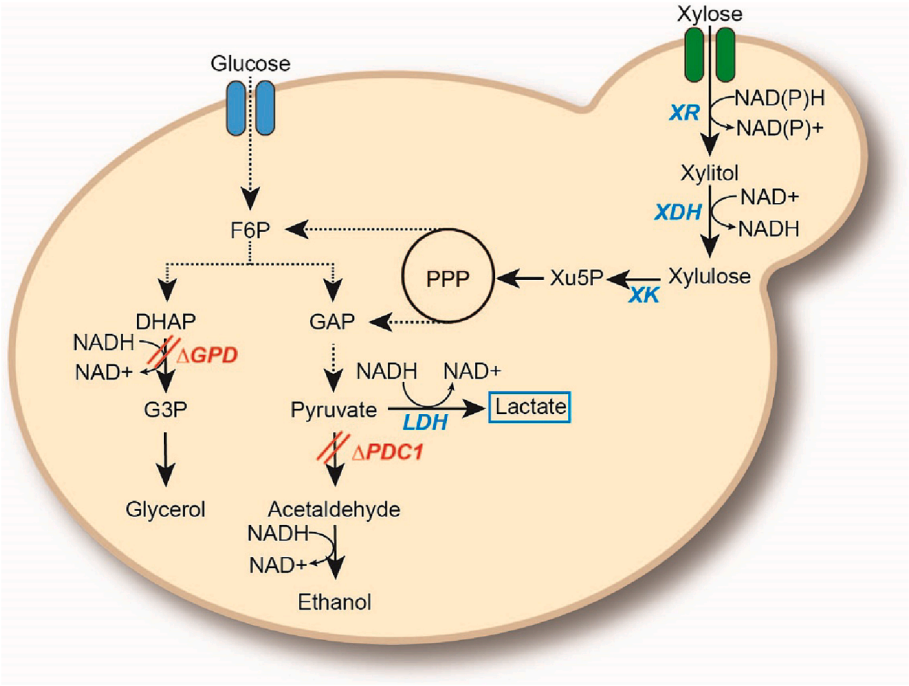

Fig. 1. Engineering the biosynthetic pathway of lactic acid in a xylose-utilizing *I. orientalis*. Solid and broken lines indicate single and multi-step metabolic reactions, respectively. Upregulated and deleted genes are shown in blue and red, respectively. Abbreviations: F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; DHAP. Dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; GPD, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; PPP, pentose phosphate pathway; XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulose kinase.

$$\text{Lactic acid production cost} = \frac{\text{Annual cost}}{\text{Annual production rate of lactic acid}} \quad (3)$$

3. Results and discussion

3.1. Construction of engineered i. Orientalis strains capable of producing lactic acid from xylose

First, an engineered *I. orientalis* capable of producing lactic acid from glucose with a high yield was constructed (Fig. 1). As *I. orientalis* SD108 strain typically produces ethanol and glycerol as main bioproducts (Lee et al., 2022), pyruvate decarboxylase (*PDC*) and glyceraldehyde-3-phosphate dehydrogenase (*GPD*) were deleted to block carbon fluxes toward ethanol and glycerol. Then, the gene encoding LmLDH (D-lactate dehydrogenase of *L. mesenteroides*) that converts pyruvate to D-lactic acid was introduced, and the resulting strain was named SD108L. The SD108XL strain, consuming xylose and producing lactic acid, was constructed by introducing *S. stipitis* XR, XDH, and XK into the SD108L strain (Fig. 1).

The phenotypes of the SD108L and SD108XL strains were examined (Fig. 2). As the production of lactic acid decreased the pH of culture media, 50 g/L calcium carbonate ($CaCO_3$), a neutralizing agent, was supplemented into the culture media. The SD108L consumed 38.7 g/L of glucose in 40 h and produced 28.7 g/L of lactic acid. The SD108XL strain consumed 44.0 g/L of xylose in 62 h and produced 28.1 g/L of lactic acid. Although the engineered *I. orientalis* strains consumed glucose faster than xylose, the cell densities (OD_{600}) were higher under xylose conditions ($OD_{600} = 14.1$) than under glucose conditions ($OD_{600} = 4.0$). While *I. orientalis* shows a Crabtree-positive response when fermenting glucose, engineered *I. orientalis* showed a weak Crabtree-positive response and produced more biomass when fermenting xylose. Another intriguing aspect of the xylose fermentation process is the production of byproducts, particularly xylitol. A previous study revealed that the ethanol-producing SD108 strain, while metabolizing xylose, produced 6 g/L of xylitol. However, the SD108XL strain produced only 1.4 g/L of xylitol (Lee et al., 2022). Similarly, when *S. cerevisiae* converted xylose to lactic acid, it produced less xylitol than when making ethanol (Turner et al., 2015). These results indicated that a robust metabolic pathway was established in *I. orientalis* for the efficient conversion of xylose to lactic acid.

3.2. Production of lactic acid from a sorghum biomass hydrolysate with pH control

It was attempted to produce lactic acid from a lignocellulosic hydrolysate using the SD108XL strain (Fig. 3). A sorghum biomass was pretreated and hydrolyzed as described in Lee et al. (2022), and the resulting hydrolysate containing 74.0 g/L of glucose and 42.3 g/L of xylose was used for producing lactic acid. Before the sorghum hydrolysate fermentation, the phenotype of the SD108XL strain was investigated using the synthetic YP medium containing 73.1 g/L of glucose and 45.4 g/L of xylose in a bioreactor. The pH was adjusted and maintained at pH 6 by adding KOH. As shown in Fig. 3A, the SD108XL strain consumed glucose and xylose completely within 49.5 h and 137 h, respectively. In the dominant glucose consumption phase (up to 49.5 h), the strain produced 50.6 g/L of lactic acid with a yield of 0.56 g lactic acid/g sugar (glucose and xylose) and a productivity of 1.02 g/L•h. After glucose depletion, an additional 20.4 g/L lactic acid was produced from xylose with a yield of 0.76 g lactic acid/g xylose and a productivity of 0.23 g/L•h.

The SD108XL strain was cultured using a sorghum hydrolysate in a shake-flask (Fig. 3B). Calcium carbonate ($CaCO_3$) was used as a neutralizing agent to prevent pH reduction caused by the accumulation of lactic acid. The SD108XL strain showed similar phenotypes as compared to the fermentation results in the synthetic medium (Fig. 3A). The SD108XL strain consumed glucose and xylose in the hydrolysates within 70 and 170 h, respectively, and produced 80.8 g/L lactic acid. The SD108XL strain produced lactic acid well by using lignocellulosic hydrolysate with the supplementation of calcium carbonate. Despite the potential presence of inhibitors like furfural and 5-hydroxymethyl-2-

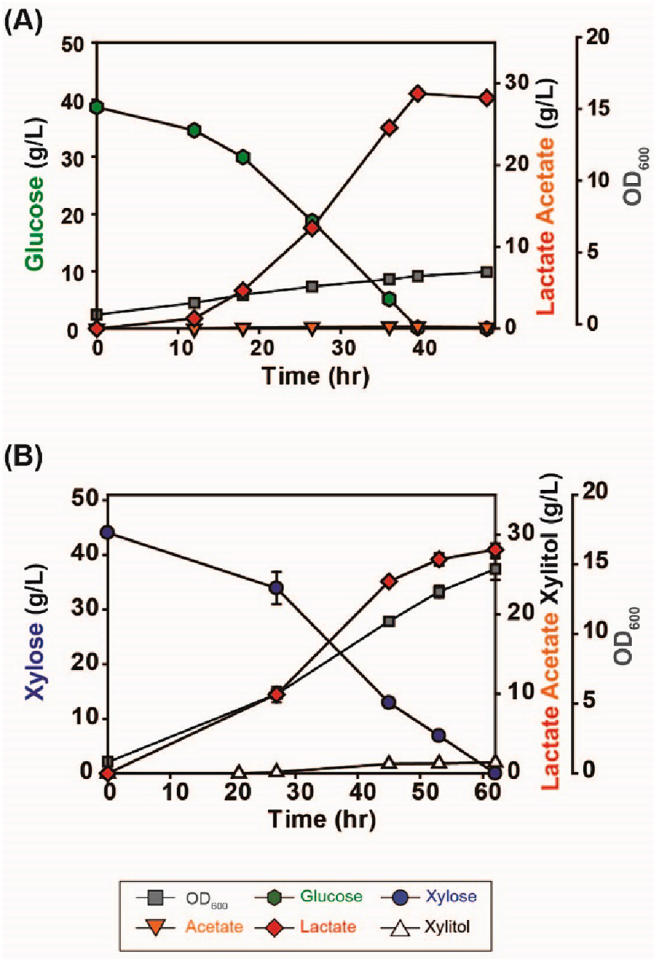

Fig. 2. Fermentation profiles of the engineered *I. orientalis* strain producing lactic acid. A) Fermentation profiles of the SD108L strain in YP medium containing 40 g/L of glucose. B) Fermentation profiles of the SD108XL strain in YP medium containing 40 g/L of xylose.

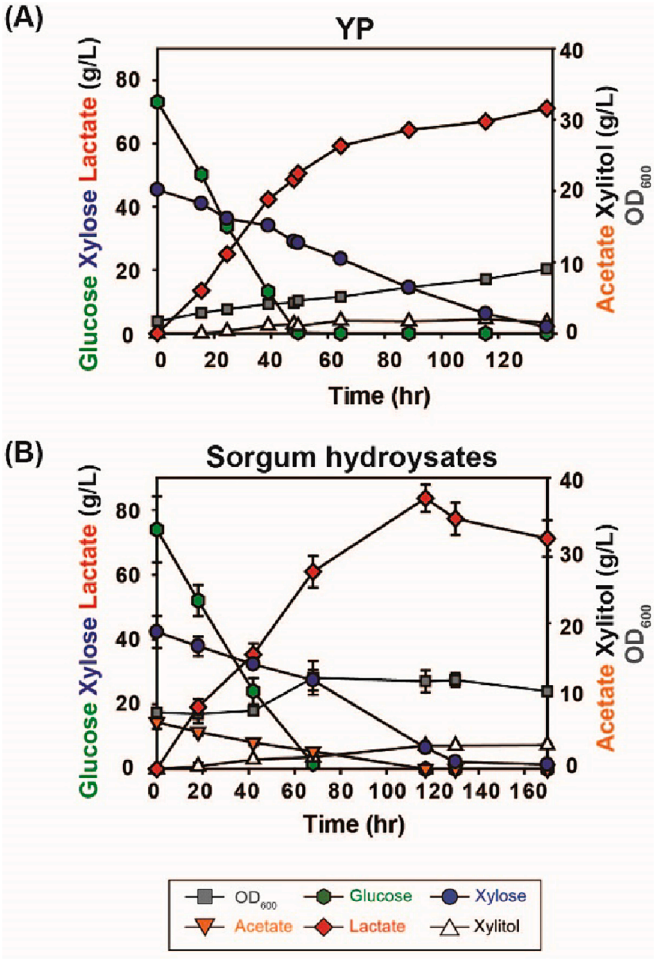

Fig. 3. Fermentation profiles of the SD108XL strain in glucose and xylose mixed sugar conditions. A) Fermentation of the SD108XL strain in DASbox with YP medium containing about 70 g/L of glucose and 40 g/L of xylose. B) Fermentation of the SD108XL strain in a 125-mL shake-flask with sorghum biomass hydrolysates. The data points of (B) represent the average of two biological duplicates, and error bars indicate standard deviations.

furaldehyde (HMF) in sorghum hydrolysates (Lee et al., 2022), *I. orientalis* appears to exhibit resilience against these inhibitory compounds.

This study aimed to produce organic acids without pH control to minimize the product recovery costs associated with neutralizing agents. However, the SD108XL strain hardly consumed glucose and xylose and produced little lactic acid without pH control (see supplementary materials). When 107.8 g/L of glucose was provided, the SD108XL consumed only 41.4 g/L of glucose and produced 32.9 g/L of lactic acid without pH regulation (see supplementary materials). When a sugar mixture of glucose (66.4 g/L) and xylose (45.6 g/L) was used, the SD108XL strain produced 30.0 g/L of lactic acid by consuming only 37.5 g/L of glucose with no xylose consumption (see supplementary materials). When more than 30 g/L of lactic acid was accumulated in the medium, the pH was around 3 (see supplementary materials). Even though *I. orientalis* is an acid-tolerant strain capable of growing well even under pH 3, high concentrations of lactic acid at pH 3 conditions seem to inhibit the growth and metabolism. These results indicate that pH control is still required for producing high levels of organic acids despite using *I. orientalis* (Park et al., 2018).

On the other hand, *I. orientalis* is typically less affected by low pH when producing non-acidic substances. Lee et al. (2022) reported that *I. orientalis* produced ethanol by consuming all glucose and xylose in sorghum biomass hydrolysates without pH control (Lee et al., 2022). It was also confirmed that *I. orientalis* consumed 40 g/L of glucose and produced 17 g/L of ethanol without pH control, while pH decreased to 2.2 (see supplementary materials). As such, *I. orientalis* appears to possess an unidentified mechanism that lowers the pH of cultures, further complicating the production of high amounts of organic acids.

3.3. Effect of lactic acid on culture buffering

Typically, similar amounts of weak acids and their conjugate base form a buffer. Based on this idea, it was hypothesized that lactic acid (a weak acid) produced by yeast during fermentation might act as a buffer for maintaining pH. In order to investigate if the lactic acid produced by cells can work as a buffer during fermentation, the SD108WT strain (ethanol-producing strain) was cultured in SC medium with 40 g/L glucose and different amounts of lactic acid. The initial pH was adjusted to 6 by KOH in all conditions (Fig. 4A). After 8.5 h of fermentation, OD_{600}, residual glucose, ethanol, and medium pH were measured. The results showed that OD_{600} and ethanol production were similar regardless of initial lactic acid concentrations. However, interestingly, the supplementation of higher amounts of initial lactic acid resulted in smaller decreases in the pH of the culture media. The culture media pH decreased to 4 in the presence of 6.4 g/L of lactic acid, while it decreased to 2 with no supplementation of lactic acid, suggesting that the supplemented lactic acid might be working as a buffer.

To further investigate this buffering effect of initially added lactic

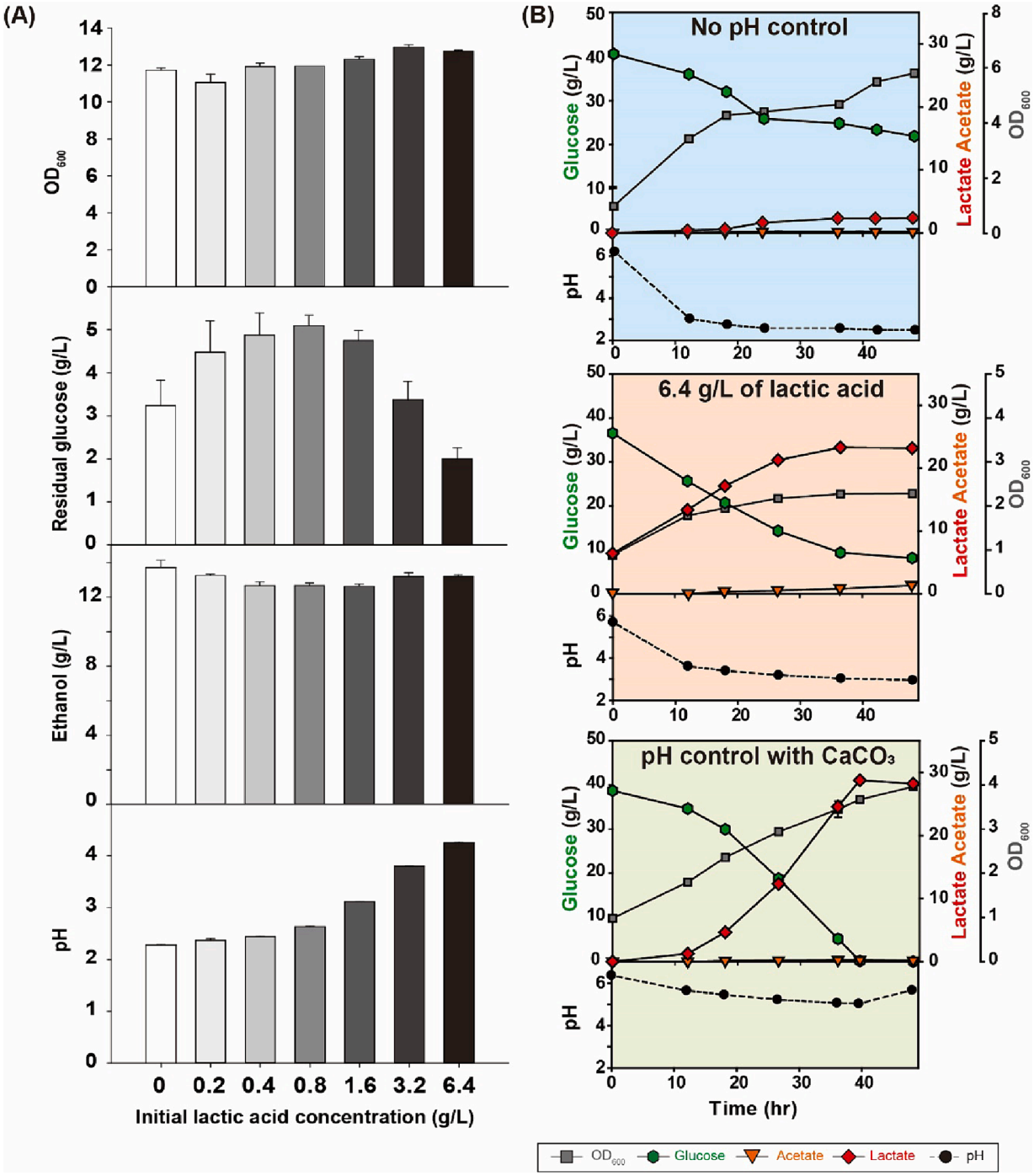

Fig. 4. Effects of lactic acid in medium on the production of organic acid in *I. orientalis*. (A) Fermentation of the SD108WT strain with different concentrations of lactic acid. (B) Fermentation of the SD108XL strain under three conditions; no pH control, 6.4 g/L of lactic acid, and pH control using $CaCO_3$. The data represent the average of two biological duplicates, and error bars indicate standard deviations.

acid, the SD108XL strain (lactic acid-producing strain) was cultured in the SC medium containing 40 g/L of glucose under three conditions: no pH control, supplemented with 6.4 g/L of lactic acid, and pH control using $CaCO_3$ (Fig. 4B). In the absence of pH control, the SD108XL strain consumed 18.6 g/L glucose and produced only 2.4 g/L of lactic acid, which can be attributed to a drastic decrease of pH to 2.5. In contrast, with pH control using $CaCO_3$, the SD108XL strain consumed all glucose and produced 28.2 g/L of lactic acid while maintaining a pH above 5. When 6.4 g/L of lactic acid was initially added to the culture medium, the pH was maintained around 3, resulting in much better glucose consumption (28.4 g/L of glucose) and lactic acid production (16.7 g/L of lactic acid) than those (10.9 g/L of glucose and 2.3 g/L of lactic acid) without lactic acid supplementation. While the SD108XL strain produced more lactic acid from glucose, the produced lactic acid and its conjugate base formed through supplementing lactic acid established a buffer system in the culture. Therefore, even as the SD108XL strain produced high amounts of lactic acid during fermentation, the pH did not decrease by Le Chatelier's Principle. Le Chatelier's Principle states that if an external change is applied to a system at equilibrium, the system will adjust itself to oppose the change until a new equilibrium is established. In the culture with lactic acid, the equilibrium exists between lactic acid (the weak acid) and its conjugate base form. When the

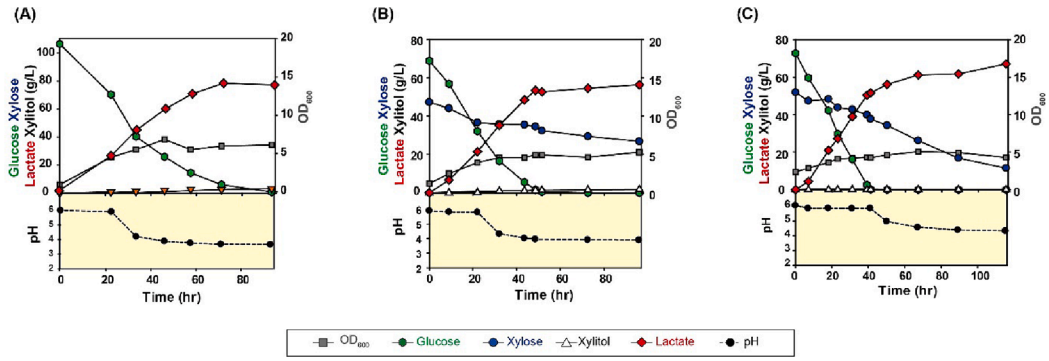

Fig. 5. Self-buffering fermentation of the SD108XL strain. (A) The fermentation of the SD108XL strain was conducted in YP medium with about 110 g/L of glucose, and the pH was maintained at 6 for the first 22.5 h. (B) The fermentation of the SD108XL strain was conducted in YP medium with 70 g/L of glucose and 40 g/L of xylose, and the pH was regulated for the first 22 h. (C) The fermentation of the SD108XL strain was conducted in YP medium with 70 g/L of glucose and 40 g/L of xylose, and the pH was regulated for the first 41 h.

SD108XL strain produced more lactic acid during fermentation, it changed the equilibrium between lactic acid and its conjugate base in the culture. Thus, the system would try to counteract this change by shifting the equilibrium towards the conjugate base form (lactate), thereby preventing a drastic decrease in pH. As a result, the pH of the culture remained relatively stable, and a substantial enhancement in fermentation performance was achieved as compared to no pH control. Additionally, in the same 6.4 g/L lactic acid conditions, the SD108XL strain producing lactic acid exhibited a lower pH (pH 3, Fig. 4B) in contrast to the SD108WT (pH 4, Fig. 4A) producing ethanol, primarily due to the lactic acid generated by the SD108XL strain.

3.4. A self-buffering fermentation strategy for producing lactic acid by engineered i. Orientalis

A self-buffering strategy was then applied to a bioreactor fermentation (Fig. 5). During the fermentation, the lactic acid produced by the SD108XL strain would be converted to the conjugate base form by initial pH control with KOH. After stopping the pH control, newly produced lactic acid would be accumulated in the medium, establishing a buffering system. When the SD108XL strain was cultured with 106.3 g/L of glucose (Fig. 5A), the pH of the culture medium was maintained at 6.0 for the first 22.5 h. During this pH control period, the SD108XL strain consumed 36.0 g/L glucose and produced 24.5 g/L lactic acid. Then, the pH control was turned off. Even though pH control was discontinued after 22.5 h, the SD108XL consumed all glucose and produced 74.0 g/L of lactic acid with a yield of 0.70 g lactic acid/g glucose while maintaining steady pH levels around 3.7. In contrast, without pH control (see supplementary materials), the SD108XL strain consumed only 41.3 g/L of glucose, while 107.8 g/L glucose was supplied and produced 32.9 g/L of lactic acid with a yield of 0.80 g lactic acid/g glucose and the pH of the culture medium decreased to 2.7.

This self-buffering strategy was also applied to mixed sugar fermentation with 68.9 g/L of glucose and 47.3 g/L of xylose (Fig. 5B). In the first 22 h of the mixed sugar fermentation, the pH of the culture medium was maintained at 6 by adding KOH until a substantial amount of lactate produced to establish a self-buffering system. When a lactic acid concentration reached 21.4 g/L after the SD108XL strain consumed 36.7 g/L of glucose and 10.7 g/L of xylose, pH control was stopped. Nonetheless, the SD108XL continued to produce lactic acid, consuming glucose completely (68.9 g/L) and a substantial amount (20.4 g/L) of xylose. The final lactic acid concentration was 56.4 g/L with a yield of 0.63 g lactic acid/g consumed sugar (glucose and xylose) while maintaining a pH of 3.9. While the self-buffering approach proved to be an effective strategy to maintain pH with minimum amounts of a neutralizing agent, the short period of pH control was not sufficient to enable efficient and rapid fermentation of xylose into lactic acid in the mixed sugar conditions (Fig. 5B). This result suggests that xylose fermentation might be more susceptible to high levels of lactic acid at low pH conditions than glucose fermentation by engineered *I. orientalis*.

There are several factors that might contribute to these observations. First, in the SD108XL strain, both *PDC* and *GPD* were deleted. *PDC* is responsible for catalyzing the conversion of pyruvate to acetaldehyde, which is subsequently converted to ethanol by alcohol dehydrogenase (ADH), oxidizing NADH into NAD^+ (Fig. 1). Consequently, if *PDC* is deleted, 1 mol of NADH is accumulated in the cytosol if 1 mol of pyruvate needs to be metabolized (Tran et al., 2023). This accumulated NADH can be utilized by LDH in the production of lactic acid. However, with the deletion of *GPD* as well, additional NADH can accumulate within the cell. This imbalance in NADH levels might impact xylose metabolism. When xylose is metabolized via XR and XDH, XR produces $NADP^+$, but XDH produces NADH, generating surplus NADH. Therefore, the deletion of *PDC* and *GPD*, causing higher NADH levels in the cytosol, might interfere with xylose metabolism negatively (Fig. 1). Conversely, in a previous study, when an engineered SD108 strain produced ethanol from xylose, there were no adverse effects on xylose consumption, even under low pH conditions (around pH 3) (Lee et al., 2022). This suggests that when *PDC* and *GPD* are not deleted, cells can maintain NADH balance, allowing for efficient ethanol production and xylose utilization even under low pH conditions (pH 3). Furthermore, low pH conditions can negatively affect the activity of the introduced xylose metabolism enzymes (XR, XDH, and XK). Considering the relatively high optimal pH of these enzymes (>6), lower pH levels of the culture could negatively impact their enzymatic activities in xylose metabolism, resulting in poor xylose utilization (Han et al., 2020).

For the efficient conversion of glucose and xylose into organic acids, it is crucial to use as much glucose and xylose as possible to achieve high titers of organic acids. As the SD108XL strain had challenges with xylose consumption at pH 3.9, it was attempted to enhance the pH by increasing the buffering capacity during fermentation. To improve the buffering capacity, higher lactic acid and its conjugate base are required. Thus, the duration of pH control was extended until glucose was completely consumed (Fig. 5C). The SD108XL strain consumed almost all glucose in 41 h and produced 51.7 g/L of lactic acid. After ceasing pH control at 41 h, the pH level dropped to 4.3, which was higher than that observed in short pH control fermentations (as depicted in Fig. 5B). Consequently, xylose consumption improved with the extended pH control, and lactic acid concentration increased to 67.1 g/L with a yield of 0.59 g lactic acid/g sugar (glucose and xylose). Even though lactic acid titer increased due to the consumption of more xylose, the yield of lactic acid (0.59 g lactic acid/g sugar) under mixed sugar conditions (72.8 g/L of glucose and 51.9 g/L of xylose, Fig. 5C) was lower than the yield (0.75 g lactic acid/g glucose) under 106.3 g/L of glucose conditions (Fig. 5A).

This result suggests that the conversion of xylose into lactic acid was inefficient—the consumed xylose might be wasted for maintenance energy—during the mixed sugar (glucose and xylose) fermentation (Fig. 5B and 5C). When xylose was used as a sole carbon source with pH control, the SD108XL strain efficiently converted xylose into biomass and lactic acid with a yield of 0.64 g lactic acid/g xylose (Fig. 2B). However, during the mixed sugar fermentation, xylose is consumed after glucose is depleted. High levels of lactic acid accumulated from glucose consumption might cause the inhibition of xylose metabolism and increase maintenance energy as well. A previous ^{13}C metabolic flux analysis study revealed that xylose metabolism activated the TCA cycle and reduced the glycolytic pathway compared to glucose utilization (Gonzalez et al., 2017). As such, when the SD108XL strain metabolizes xylose after glucose depletion, xylose appears to be primarily used for activating the TCA cycle, generating additional maintenance energy to counter unfavorable conditions rather than producing biomass and lactic acid. Consequently, despite implementing the self-buffering strategy, the yield and productivity of lactic acid on xylose during the stationary phase remained low. Therefore, further advancements in bioprocessing and strain engineering are needed to enhance lactic acid production from glucose and xylose.

Nevertheless, the engineered *I. orientalis* strain and self-buffered fermentation approach showed significant potential for the industrial production of organic acids from glucose and xylose. Traditionally, *S. cerevisiae* has been mainly studied for the production of lactic acid. While these studies reported higher titers and yields, they utilized only glucose as a substrate and neutralizing agents throughout the fermentation process (Mitsui et al., 2020; Song et al., 2016; Watcharawipas et al., 2021). Conversely, in this study, lactic acid production was achieved from a mixed of glucose and xylose, utilizing a self-buffering strategy at a pH of 4.3. This pH level is generally lower than that reported in other studies, with the exception of the work by Baek et al., (2017), who enhanced a lactic acid production capability through adaptive laboratory evolution (Baek et al., 2017). In their approach, the engineered *S. cerevisiae* was gradually exposed to increasing levels of lactic acid. This led to the strain that produced lactic acid with a titer of 83 g/L and a yield of 0.83 g/g glucose, 24 % and 38 % higher than the results of this study by *I. orientalis*, respectively. Consequently, it is expected that employing an adaptive evolution approach with the SD108XL strain could further improve lactic acid production efficiency, even under more acidic conditions.

3.5. Techno-economic analysis of the self-buffering strategy for the production of lactic acid by i. Orientalis

A techno-economic analysis (TEA) was conducted to compare the economic viability of a self-buffering strategy against the traditional pH control method in lactic acid production. The lactic acid production process encompasses several stages, including feedstock preparation, liquefaction, saccharification, fermentation, acidification, gypsum separation, and purification (see supplementary material) (Manandhar & Shah, 2023; Marchesan et al., 2021). In this study, the analysis mainly focused on the fermentation with pH control, acidification, and gypsum separation stages (Gezae Daful & Görgens, 2017). The process was modeled and simulated by Superpro Designer v9.5 (see supplementary material). The experimental data (the fermentation dynamics, lactic acid yields, and consumption of carbon sources) were used to simulate the processes (referenced in Fig. 3A and 5C, respectively). In the TEA, calcium hydroxide ($Ca(OH)_2$) was selected over KOH (used experimentally in this study) for neutralization during fermentation to align with conventional practices. In the acidification step, sulfuric acid (H_2SO_4) facilitated the conversion of lactate to lactic acid, generating gypsum as a byproduct. Gypsum removal was achieved using hydrocyclones, followed by washing to extract residual lactic acid. Key process parameters and assumptions are also summarized in the supplementary material.

Table 2
TEA results for lactic acid production by *I. orientalis* through pH control and self-buffering Methods.

Cost \ Conditions	pH control[a]	self-buffering[b]
Annualized capital cost ($/yr)	26,003,480	22,918,580
Annual operating cost ($/yr)	18,919,338	17,328,638
Annual cost ($/yr)	44,922,818	40,247,218
Lactic acid productivity (kg/yr)	1,000,000	1,000,000
Lactic acid production price ($/kg)	44.9	40.2

[a] The data are referred to in Fig. 3A.
[b] The data are referred to in Fig. 5C.

The pH control and self-buffering strategies were simulated with the goal of producing 1,000 metric tons (MT) of lactic acid. The TEA result revealed that the self-buffering method required significantly lesser quantities of $Ca(OH)_2$ and H_2SO_4, specifically 100 MT less of $Ca(OH)_2$ and 134 MT less of H_2SO_4 compared to the conventional pH control strategy (see supplementary material). This strategic reduction in chemical use led to decreased gypsum production. Consequently, there was a reduced need for washing water, with the process consuming 547 MT less water for gypsum washing in the self-buffering process. Moreover, the lower gypsum output also led to a decrease in the energy required for its separation in hydrocyclones, resulting in a significant energy saving of 507,851 kWh. This efficiency in energy use for gypsum separation further underscores the environmental and operational cost benefits of the self-buffering strategy in lactic acid production.

The self-buffering approach not only resulted in a leaner process flow with reduced reaction volume compared to the pH-controlled scenario but also manifested in lower capital and operational costs for the same volume of lactic acid production. Accordingly, the production cost under self-buffering conditions was $40.2/kg, indicating a cost advantage of 10 % over the pH control method (Table 2). These findings highlight the economic and environmental benefits of a self-buffering strategy for lactic acid production.

4. Conclusion

This study demonstrated the cost-effective production of lactic acid using the acid-tolerant yeast, *I. orientalis*. The engineered strain, SD108XL, efficiently processed glucose and xylose in lignocellulosic hydrolysates but struggled without pH control. To address this issue, a self-buffering strategy was employed to maintain a pH of around 4, enhancing sugar consumption and lactic acid production while minimizing the need for neutralizing agents. The techno-economic analysis revealed that the self-buffering strategy could reduce production costs, demonstrating the feasibility of *I. orientalis* for efficient organic acid production.

CRediT authorship contribution statement

Ye-Gi Lee: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Nam Kyu Kang:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chanwoo Kim:** Methodology, Investigation, Formal analysis. **Vinh G. Tran:** Methodology. **Mingfeng Cao:** Methodology. **Yasuo Yoshikuni:** Writing – review & editing, Supervision. **Huimin Zhao:** Writing – review & editing, Supervision. **Yong-Su Jin:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130641.

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