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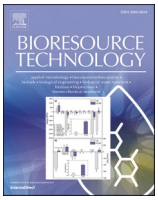

pH shifting adaptive evolution stimulates the low pH tolerance of *Pediococcus acidilactici* and high L-lactic acid fermentation efficiency

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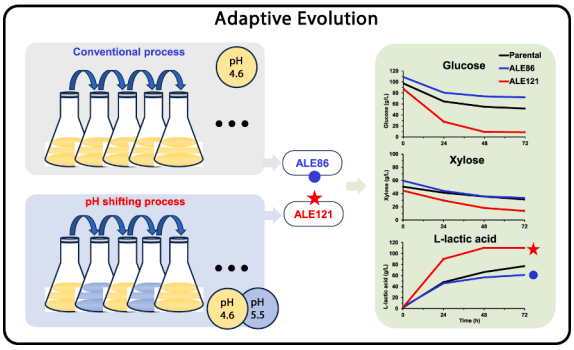
HIGHLIGHTS

- pH shifting adaptive evolution improved the acid-tolerance of lactic acid bacteria.
- The evolved strain showed better L-lactic acid fermentation performance.
- The final cellulosic L-lactic acid generation was improved by 42.9% at pH 4.6.

GRAPHICAL ABSTRACT



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ABSTRACT

L-lactic acid fermentation at low pH reduces the use of neutralizers during fermentation and the generation of solid wastes in purification processes. Most lactic acid bacteria exhibit weak tolerance and poor cell viability at low pH. This study proposes a pH shifting adaptive evolution method to improve the low-pH tolerance of an engineered *Pediococcus acidilactici* strain. In the first stage, cells were cultured at a moderate pH to maintain the cell viability, then shifted to a low pH to enhance low-pH tolerance. Long-term pH shifting evolution culture of the engineered *P. acidilactici* between the moderate and low pH resulted in a 43 % increase in L-lactic acid production at pH 4.6 (110.4 g/L) and a 2.1-fold increase at pH 4.4 (80.7 g/L) compared to the parental strain when using wheat straw as a feedstock. This pH-shifting adaptive evolution strategy provides an effective tool for improving the low-pH tolerance of lactic acid bacteria.

1. Introduction

The accumulation of L-lactic acid during L-lactic acid fermentation inevitably leads to a decline in pH. To maintain the proper pH levels, neutralizers such as calcium hydroxide or calcium carbonate are added. This result in increased operation costs and the generation of calcium sulfate solid wastes in the subsequent purification steps (Marchesan et al., 2021). If the L-lactic acid fermentation strain can tolerate a low pH environment, the need of neutralizers and the generation of solid wastes could be significantly reduced, or even potentially eliminated (Pangestu

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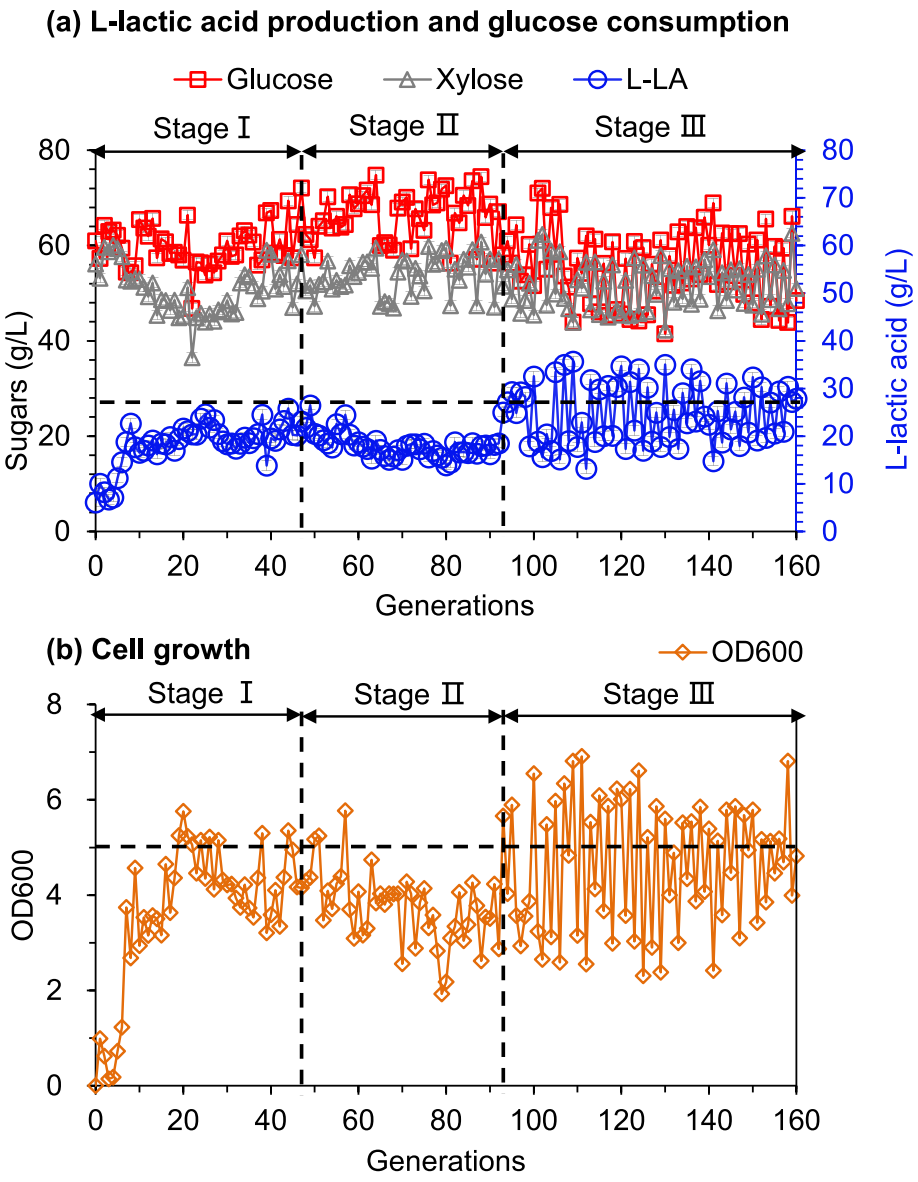

Fig. 1. Low pH adaptive evolution of *P. acidilactici* ZB220. (a) L-lactic acid production and sugar consumption; (b) Cell growth. The initial medium pH of Stage I and II were 4.8 and 4.6. In Stage III, transfers were conducted alternately in mediums of pH 5.5 and pH 4.6.

et al., 2022; Zhang et al., 2023a).

Lactic acid bacteria, particularly *Pediococcus acidilactici*, are among the most promising strains for L-lactic acid production due to their high yield (over 90 %), better temperature tolerance (42–48 °C), and strong resistance to inhibitors (He et al., 2022; Li et al., 2024). The latest developed engineered lactic acid bacteria strains can completely and coordinately convert lignocellulose-derived fermentable sugars into high-purity L-lactic acid products suitable for use as polylactic acid (PLA) monomer (He et al., 2022). However, the growth and cell viability of lactic acid bacteria at low pH are relatively weak, with a conventional fermentation pH value of around ~5.5 (Papadimitriou et al., 2015; Zhao et al., 2013). Currently, various methods are employed to improve the low pH tolerance of lactic acid bacteria. Acidic adaptive evolution and metabolic modification are wildly used techniques, for example, introducing genes such as *recA* or *dnaK*, which are related to DNA or protein damage repair, or improving the ATP synthesis to pump out more H^+ and maintain the stability of intracellular pH (Yan et al., 2022; Zhang et al., 2023b). Despite these efforts, no significant progress has been achieved (Zhu et al., 2024).

In this study, a pH-shifting adaptive evolution strategy was applied to the engineered lactic acid bacterium strain *Pediococcus acidilactici* ZB220, which is characterized by its highly cellulose chiral lactic acid-producing, temperature tolerance, high resistance to lignocellulose system, and ability to metabolize all sugars. *P. acidilactici* ZB220 was alternately cultured between a conventional pH of 5.5 and an acidic environment of pH 4.6 to impose low-pH stress while allowing for the restoration of cell viability. The results indicated that the pH shifting strategy effectively improved the low pH tolerance and the cellulosic L-lactic acid fermentation performance of the engineering lactic acid bacteria under low pH conditions.

2. Materials and methods

2.1. Strains

Pediococcus acidilactici ZB220 (CGMCC M2023151), which modified by metabolic engineering and adaptively evolved, can completely and coordinately convert lignocellulose derived fermentable sugars, was employed as the L-lactic acid fermentation strain using dry acid pretreated and biodetoxidfied wheat straw as the feedstock (Qiu et al.,

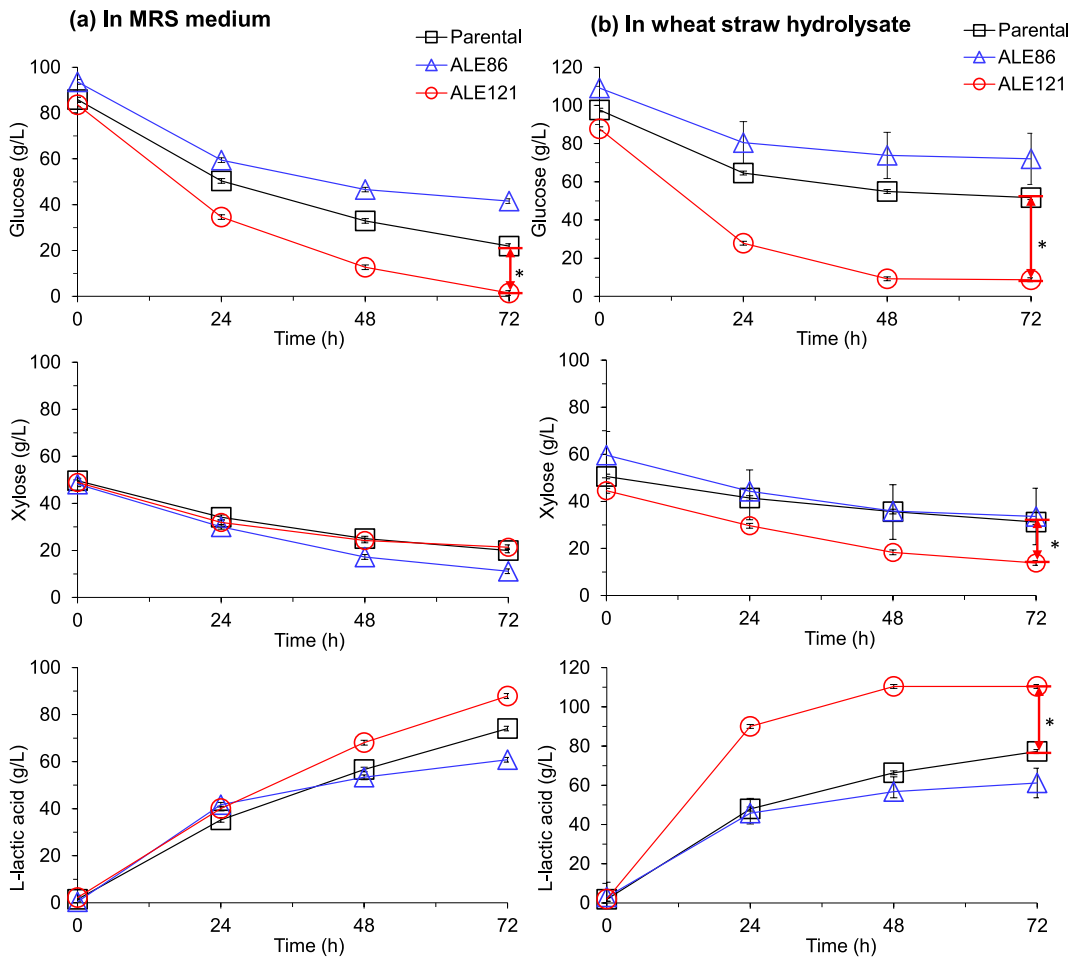

Fig. 2. L-lactic acid fermentation in MRS medium (a) and wheat straw hydrolysate (b) by adaptive evolution strains. The fermentations were conducted in 3 L bioreactor at 42 °C, 300 rpm for 72 h, and at 30 % (w/w) solids loading of pretreated and biodetoxified wheat straw hydrolysate. The pH naturally drops rapidly to 4.6 and is maintained at this value by the automatic addition of 25 % (w/w) $Ca(OH)_2$. Asterisk symbol (*) indicated a significant difference between two groups (p-value < 0.01) according to *t*-test analysis. All experiments were conducted in duplicate.

2018). The strain was activated and cultured in a simplified man-rogosa-sharp (MRS) medium to serve as the seed broth(He et al., 2022).

Paecilomyces variotii FN89 (CGMCC #17665) was used as the biodetoxification strain, cultured on potato-dextrose-agar medium as described in our previous publication (Zhang et al., 2021).

2.2. Reagents, feedstock, and biorefinery operations

Commercial cellulase enzyme Cellic CTec 3 was purchased from Novozymes China (Beijing, China). Other reagents were obtained from Titan Scientific Co. (Shanghai, China).

Wheat straw was pretreated using dry acid pretreatment methods (Liu et al., 2018). The pre-hydrolysis was conducted at a solids loading of 30 % (w/w), 50 °C, and 200 rpm for 12 h (Zhang et al., 2024). Then biodetoxification was conducted by inoculating the biodetoxification seed at 37 °C, 600 rpm, and aeration of 1 vvm for 20 h to degrade the inhibitors (Zhang et al., 2021).

2.3. Adaptive evolution

Low pH adaptive evolution was performed in a 100 mL flask containing 20 mL simplified MRS medium with mixed sugars. The low pH environment was established by adding L-lactic acid to the medium to adjust the initial pH. The strain was transferred at a 10 % (v/v) inoculation volume every 24 h, and the pH was adjusted to the initial value by adding 5 M NaOH solution every 8 h. The entire adaptive evolution process comprised three stages, the initial pH values for Stage I and Stage II were 4.8 and 4.6, respectively. Subsequently, a pH-shifting strategy was implemented in Stage III, involving alternate transfers every 24 h between a regular medium (pH 5.5) and an acidic medium (pH 4.6). Samples of each generation were collected before each transfer.

2.4. L-lactic acid fermentation

L-lactic acid fermentations were conducted in a 3L bioreactor at 42 °C, 300 rpm, and with an inoculum size of 10 % (v/v). The simultaneous saccharification and co-fermentation (SSCF) was carried out using wheat straw hydrolysate (He et al., 2022). The fermentation medium and the wheat straw pre-hydrolysate slurry both contained 100 g/L of glucose and 50 g/L of xylose.

2.5. Analysis methods

Sugars and L-lactic acid were analyzed by the HPLC method (Qiu et al., 2018).

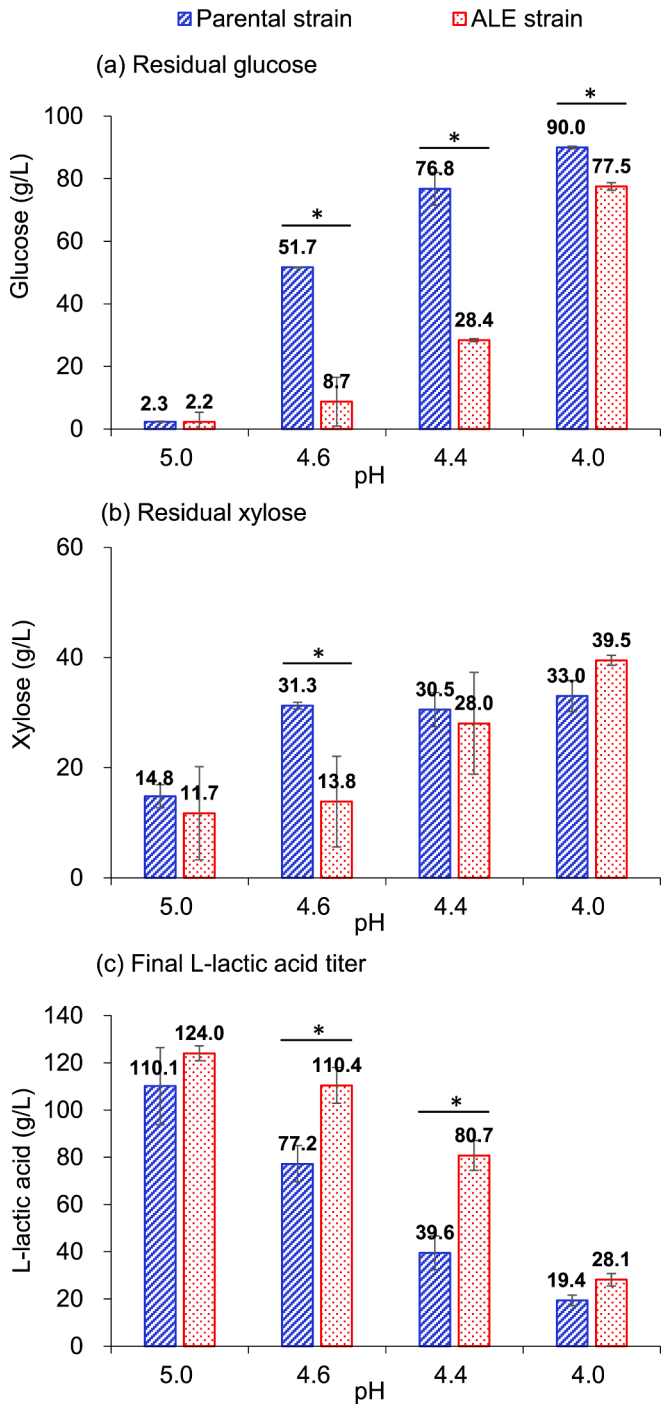

Fig. 3. Evaluation of L-lactic acid fermentation at low pH by pH shifting adaptive evolved ALE121 using the pretreated and biodetoxified wheat straw ydrolysate at 30 % (w/w) solids loading, 42 °C, 300 rpm for 72 h. Asterisk symbol (*) indicated a significant difference between two groups (p-value < 0.01) according to *t*-test analysis. All experiments were conducted in duplicate.

3. Results and discussion

3.1. pH shifting adaptive evolution to improve the cell viability of engineered Pediococcus acidilactici

Adaptive evolution is a simple and effective method for acquiring target properties or functions by imposing specific pressures (Shi et al., 2022). *P. acidilactici* ZB220 was firstly subjected to adaptive evolution under acidic conditions to improve its performance in cellulose lactic acid fermentation at low pH value (Fig. 1). Significant decreases in sugar consumption and L-lactic acid generation was observed when pH was reduced to 4.8 (**Fig. S1**, see Supplementary Material). Therefore, pH 4.8 was selected as the starting pH value of the adaptive evolution culture.

In Stage I of the adaptive evolution for *P. acidilactici* ZB220, the initial pH level of the culture medium was adjusted to 4.8 by adding lactic acid to create acidic screening pressure. Fig. 1 shows that the cell growth gradually increased after ten transfers with enhanced L-lactic acid production from the initial period of adaptive evolution. In Stage II, the initial pH value was reduced to 4.6 from 4.8, and weaker cell growth was observed, along with a reduction in L-lactic acid production and sugars consumption. To restore cell viability, a pH shifting strategy was introduced in Stage III by alternate transfer between the moderate pH of 5.5 and the low acidic pH of 4.6. The regular pH culture allowed the cells of *P. acidilactici* ZB220 to periodically recover from the harsh acidic condition for improving the cell viability and metabolic activity, then the acidic low pH culture was introduced as low pH tolerance adaptive evolution. The pH values at each transfer were measured ranging from 3.9 to 4.1. Fig. 1 showed that *P. acidilactici* ZB220 grew better in each recovery interval and maintained its L-lactic acid production capacity when shifted to the low pH condition. The final cell growth, sugar consumption, and L-lactic acid generation of the evolved strain at pH 4.6 were close to those at pH 4.8. These changes during the evolutionary process suggest that pH shifting adaptive evolution was effective in recovering the cell viability and fermentative capacity of the evolved strain.

3.2. L-lactic acid fermentation evaluation of adaptive evolved cells

The two evolved strains after pH shifting adaptive evolution—ALE86 (isolated in Stage II, before pH shifting adaptive evolution) and ALE121 (isolated in Stage III, after the pH shifting adaptive evolution)—were evaluated for L-lactic acid fermentation at pH 4.6. The evaluations were conducted using both pure sugars (simplified MRS medium) (Fig. 2**a**) and wheat straw hydrolysate after dry biorefining processing (Fig. 2**b**).

Fig. 2**a** showed that when pure glucose and xylose were used as carbon sources, the glucose consumption and L-lactic acid production of ALE86 (without pH shifting adaptive evolution) were reduced, while xylose consumption slightly increased at pH 4.6 compared to the parental strain. In contrast, for ALE121 (after pH shifting adaptive evolution), glucose consumption was increased by 28.5 %, xylose consumption remained consistent, and L-lactic acid production increased by 42.9 % at pH 4.6 compared to the parental strain.

Fig. 2**b** showed that the low pH (at pH 4.6) tolerance of the two evolved strains was further enhanced when practical wheat straw was used as the carbon feedstock. For ALE86, the glucose consumption was reduced by 19.2 %, xylose consumption slightly increased, and L-lactic acid production decreased by 22.8 %. For ALE121, glucose consumption was increased by 72.1 %, xylose consumption was increased by 58.6 %, and L-lactic acid generation was increased by 44.0 % at pH 4.6 compared to the parental strain.

Cellulosic L-lactic acid fermentation of the evolved strain ALE121 was conducted across a broad pH range from 4.0 to 5.5 (Fig. 3 **and Fig. S2**, see Supplementary Material). ALE121 showed increased L-lactic acid production compared to the parental strain under each tested pH condition. Notably, at pH 4.6 and 4.4, ALE121 improved glucose consumption by 83.1 % and 63.0 %, respectively, compared to the parental strain, with only 13.8 g/L of xylose remaining at pH 4.6. Regarding final L-lactic acid production, ALE121 showed a significant increase, producing 110.4 g/L at pH 4.6, corresponding to a 42.9 % improvement over the parental strain. The L-lactic acid yield was 0.61 g per gram of sugar consumed and the productivity was 1.50 g/L/h, compared to 0.41 g/g and 1.04 g/L/h of the parental strain, respectively. Finally, at pH 4.4, the final L-lactic acid titer also reached 80.7 g/L which was 2.1 times higher than that of the parental strain. The optimal fermentation pH for *P. acidilactici* ZB220 is 5.5 (Qiu et al., 2018), and fermentation

performance is acceptable even when the pH is reduced to 5.0. The evolved strain exhibits well fermentation performance at pH 4.6 which is comparable to that at pH 5.0. These results indicate that the pH shifting adaptive evolution not only improved the acid tolerance of *P. acidilactici* ZB220 but significantly enhanced the cellulosic L-lactic acid fermentation performance at pH 4.6, which is approximately 1.0 pH unit lower than the conventional fermentation pH. For very low pH values approaching the pKa of L-lactic acid (~3.78), the pH shifting adaptive evolution should still be effective, and further long-term pH shifting adaptive evolution is under investigation.

Studies on low pH lactic acid fermentation predominantly feature acid-tolerant yeasts as the fermentation strains. These yeasts exhibit excellent acid resistance and high lactic acid production by metabolic engineering (Liu et al., 2023). However, most yeast strains exhibit poor adaptability to lignocellulose system, including the temperature condition and inability to simultaneously utilize lignocellulose derived fermentable sugars. In contrast, this study applied pH shifting evolution strategy on lactic acid bacterium *P. acidilactici* ZB220, which can perfectly adapt to the production of cellulosic L-lactic acid from lignocellulosic feedstocks. Through this shifting evolution strategy, we acquired an evolved strain that not only tolerates low pH conditions but also exhibits high L-lactic acid fermentation performance.

Adaptive evolution often involves harsh conditions for screening and evolving target strains with specific characterizations and functions (Shi et al., 2022). However, prolonged screening pressure may simultaneously bring certain negative effects to the strain, such as inhibiting growth or reducing metabolic viability, as mentioned in this study. Shifting adaptive evolution, on the other hand, creates a recovery period during the evolution process, retaining the screening pressure while allowing microorganisms to recover under relatively mild conditions. The evolved strains may acquire new characteristics and retain their inherent excellent traits simultaneously. Therefore, the strategy of shifting adaptive evolution may also be applied to other adaptive evolutionary processes, creating a recovery interval and achieving more stable or efficient evolutionary outcomes.

4. Conclusion

In this study, an evolved strain was obtained by pH shifting adaptive evolution with the advantages of high cellulosic lactic acid fermentation performance at lower pH. The shifting adaptive evolution strategy is a correct and effective attempt for low pH lactic acid fermentation.

CRediT authorship contribution statement

Zhibin Li: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Lingxiao Zhang:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Bin Zhang:** Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition. **Jie Bao:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, 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.

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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.2024.131813.

Data availability

Data will be made available on request.

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