Chapter 1

Introduction

1.1 Background

Lactic acid is one of the most important platform chemicals used in the production of polylactic acid (PLA), a biodegradable polymer that serves as a sustainable alternative to petroleum-based plastics (Karamanlioglu et al., 2017). Owing to increasing environmental concerns regarding plastic waste, the demand for PLA has grown rapidly over the past decade. Global PLA production reached approximately 800,000 tonnes by 2020, with a market value of around USD 5.2 billion and projected to exceed USD 9.5 billion by 2027 (Zwiercheczewski et al., 2022; Joseph et al., 2023). Beyond its role as a precursor for PLA, lactic acid is widely used in food, bioplastic, and biomedical industries. PLA is extensively applied in packaging materials, mulch films, rigid containers, single-use food service products, textile fibers, and 3D-printing filaments (Swetha et al., 2023). In addition, pure lactic acid is used in biomedical applications such as surgical sutures, tissue scaffolds, implants, and controlled-release systems (Hussain et al., 2024), as well as in fermented and probiotic food products.

Industrial lactic acid production is currently dominated by lactic acid bacteria (LAB), which are efficient producers but suffer from several drawbacks. Many LAB strains are highly sensitive to acidic conditions and therefore require neutralizing agents during fermentation, increasing downstream processing costs and generating undesirable by-products (Rahman & Sonomoto, 2016). In addition, LAB often requires complex and expensive growth media, while contamination and bacteriophage infections can reduce process stability (Huang et al., 2023; Jang et al., 2021). These limitations highlight the need for alternative microbial hosts capable of supporting efficient and economically viable lactic acid production.

One promising alternative host is *Komagataella phaffii* (formerly *Pichia pastoris*), a methylotrophic yeast capable of utilizing methanol as its sole carbon source (Vijayakumar & Venkataraman, 2024).

This characteristic is particularly attractive for industrial biotechnology because methanol is an inexpensive substrate and a strong inducer of gene expression in *K. phaffii*. The yeast is widely used for recombinant protein production due to its tightly regulated alcohol oxidase 1 (*AOX1*) promoter, high cell density cultivation capability, and ability to perform eukaryotic post-translational modifications (Bustos et al., 2022; Lv & Cai, 2025). The robustness of *K. phaffii* has led to its adoption by a lot of biotechnology, pharmaceutical, vaccine, and food companies worldwide (Moraes et al., 2024). Currently, the commercial products produced using this platform are mainly recombinant proteins, include recombinant insulin, human serum albumin, COVID-19 vaccines, VHH antibodies, and monoclonal antibodies (Barone et al., 2023; Dalvie et al., 2024).

Beyond recombinant protein production, *K. phaffii* has increasingly been explored as a platform for metabolic engineering and the biosynthesis of various organic compounds, including malic acid, itaconic acid, xylonic acid, etc. (Carneiro et al., 2022). Notably, engineered *K. phaffii* strains have achieved itaconic acid titers of approximately 55 g/L within five days, with subsequent improvements reaching 103.8 g/L at pH 5.5 and 50.4 g/L at pH 3.5 (Severinsen et al., 2024; Wei et al., 2025). These achievements demonstrate the potential of *K. phaffii* as an industrial microbial platform extending beyond recombinant protein expression.

Despite its industrial versatility, *K. phaffii* does not naturally produce lactic acid as a major fermentation product. However, its native metabolism can potentially be redirected toward lactate production through metabolic engineering. One strategy is to disrupt the native alcohol fermentation pathway while introducing the lactate dehydrogenase pathway, thereby redirecting pyruvate from ethanol production toward lactic acid biosynthesis. Such targeted metabolic pathway modifications are now feasible through CRISPR/Cas9 genome editing technology, which enables precise insertion, deletion, and replacement of genes within the *K. phaffii* genome. Therefore, this study aims to establish a CRISPR/Cas9-based genome editing strategy to insert the *Pediococcus acidilactici ldh-l*

gene (*Paldh*) into the *adh2* locus of *K. phaffii*, thereby disrupting alcohol dehydrogenase activity and redirecting alcohol fermentation toward lactic acid production. Successful implementation of this strategy may provide a foundation for developing *K. phaffii* as a novel host for sustainable lactic acid and other organic acid production.

1.2 Objective

The objective of this research is to develop a genetically engineered *K. phaffii* strain with lactate fermentation activity for lactic acid production. This will be achieved by

(1) Construction of donor DNA consisting of *Paldh* coding sequence flanked by promoter and terminator region of *Kpadh2* gene.

(2) Construction of CRISPR/Cas9 plasmid harboring the sgRNA for knocking out the native *Kpadh2* gene.

(3) Co-transformation of donor DNA and CRISPR/Cas9 plasmid for integration of *Paldh* expression cassette into the *K. phaffii* genome.

1.3 Hypothesis

Null Hypothesis (H_0):

CRISPR/Cas9-mediated genome editing does not result in knockout of the *adh2* gene or successful integration of the *Paldh* expression cassette into the genome of *K. phaffii*.

Alternative Hypothesis (H_1):

Simultaneous CRISPR/Cas9-mediated knockout of the native *Kpadh2* gene and homologous integration of the *Paldh* expression cassette into the *K. phaffii* genome will successfully remove the *Kpadh2* gene and integrate the *Paldh* gene into *K. phaffi* genome.