Chapter 1

Introduction

1.1 Background

Lactic acid serves as a valuable monomer that is notably used for the production of biodegradable polymers with higher mechanical qualities, as well as thermal and hydrolysis resistance (Yamada et al., 2019). As a byproduct of various lactic acid bacteria, it is heavily utilized in the food and pharmaceutical industries, resulting in a high market demand. The conventional microbial production of lactic acid significantly lowers pH, which is not optimal for the microorganism. In order to maintain neutral pH needed for microbial growth, neutralizing agents like gypsum are added, thus producing gypsum waste. This necessitates the use of neutralizers, which further adds up to the production costs (Wang et al., 2025).

The biosynthesis of lactic acid occurs through the conversion of pyruvate, catalyzed by lactate dehydrogenase (LDH). LDH, an oxidoreductase enzyme, catalyzes the conversion of lactate into pyruvate while reducing NAD^+ and NADH. This conserved enzyme enables energy production via the anaerobic metabolism of glucose in oxygen-depleted environments (Khan et al., 2020). It accelerates the interconversion of pyruvate to lactate and NADH to NAD^+ by 14 times and is regulated by transcription factors, substrates, and allosteric modulators (Papaneophytou et al., 2021). One of the microorganisms that utilizes LDH is *Lactiplantibacillus plantarum*, a gram positive, heterofermentative, catalase negative bacteria widely used for food fermentation processes and recognized for its preservative qualities due to lactic acid production (Seddik et al., 2017). However, as a heterofermentative organism, *L. plantarum* does not fully utilize its carbon metabolism for lactic acid production, instead producing other byproducts, such as acetic acid. Furthermore, optimizing lactic acid production in bacteria requires the maintenance of appropriate pH level, as production is

adversely affected by the pH of the culture (Siegumfeldt et al., 2000). This highlights the need for a robust organism capable of producing high amounts of lactic acid and is tolerant to the drop in pH.

One of these robust organisms is yeast, as it is known for its ability to tolerate an acidic environment, eliminating the need for neutralizers while also utilizing a diverse range of carbon substrates. However, it does not have a native lactic acid pathway, which means that the *LDH* would need to be integrated within the system. A potential yeast for lactic acid production is *Komagataella phaffii*, an emerging model organism that has been well-researched for bioproduction of an organic compound. Its suitability for genetic engineering is highlighted by its rapid growth, ease of cultivation, robust inducible promoter, and notable thermo- and osmo-tolerance, coupled with established genetic modification tools for gene recombination (Bernauer et al., 2021). It has the potential to reach higher yields of lactic acid production using heterologous expression through engineered yeast strains through metabolism redirection, optimization of gene expression, and refined control over carbon utilization via genetic engineering approach (Tsaruk et al., 2025).

Previous research has successfully integrated the lactic acid pathway within yeasts for lactic acid production. A study done by Inoue et al. (2025) successfully produced acid in *K. phaffii* by introducing four copies of D-lactate dehydrogenase from *Leuconostoc mesenteroides*. The genes insertion into the ribosomal DNA (rDNA) locus and expression under the control of *AOX1* promoter and terminator effectively facilitate lactic acid production. Another study used a different LDH integration method alongside a metabolic engineering method called gene swapping. Park et al. (2018) replaced *PDC* of *Pichia kudriavzevii* with *LDH* isolated from *L. plantarum* using *URA3* pop-out system. The PDC itself was swapped in order to redirect the pyruvate from the ethanol fermentation pathway. It resulted in a maximum D-Lactic acid production at 135 g/L using low-pH fed-batch fermentation with neutralization. Another metabolic engineering method was utilized by Baek et al. (2015), involving the deletion of alcohol dehydrogenase and pyruvate decarboxylase. The study found that the

engineered yeast achieved higher lactic acid production, as previous strains achieved production of 38.3 g/L of D-lactic acid while engineered strains produced 48.9 g/L of D-lactic acid. These findings highlight the importance of metabolic engineering for optimized production within yeast. However, previous research has not explored the possibilities of gene swap within the *ADH2* locus of *K. phaffii* using the *LDH* of *L. plantarum*, especially using the CRISPR/Cas9 system to facilitate transgene integration.

This study aims to provide the preliminary research of heterologous expression of lactic acid in *K. phaffii* by integrating *LDH* of *L. plantarum* in the *ADH2* locus of *K. phaffii*. This is done through construction of a donor DNA fragment containing *LDH* coding sequence surrounded by the upstream and downstream of *ADH2* gene in *K. phaffii*, alongside the assembly and integration of sgRNA for the purposes of knocking out the *ADH2*. The *LDH* coding sequence of *L. plantarum* will also be evaluated for heterologous expression in *K. phaffii* using In silico characterization.

1.2 Objective

The primary aim is to isolate *Lactiplantibacillus plantarum LDH* and the upstream and downstream of *ADH2* of *Komagataella phaffii* using PCR, and analyze *LDH* using In silico characterization. Then construct donor DNA consisting of an *LDH* coding sequence isolated from *L. plantarum* and the *ADH2* gene upstream and downstream sequences from *K. phaffii*, alongside Cas plasmid for the heterologous expression within *K. phaffii* using OE-PCR and Golden Gate Assembly, verified by gel electrophoresis and colony PCR.

1.3 Hypothesis

For gene isolation :

- Null Hypothesis (H_0): The *LDH* coding sequence of *L. plantarum*, and the upstream and downstream of *ADH2* of *K. phaffii* can not be isolated.

- Alternative Hypothesis (H_1): The *LDH* coding sequence of *L. plantarum*, and the upstream and downstream of *ADH2* of *K. phaffii* can be isolated.

For assembly of donor DNA cassette and Cas plasmid:

- Null Hypothesis (H_0): The *LDH* of *L. plantarum* can not be integrated into a donor DNA cassette meant for the gene swap in *ADH2* locus in *K. phaffii* and sgRNA of *K. phaffii ADH2* can not be assembled and integrated into a CRIS*Pi* plasmid
- Alternative Hypothesis (H_1): The *LDH* of *L. plantarum* can be integrated into a donor DNA cassette meant for the gene swap in *ADH2* locus in *K. phaffii* and sgRNA of *K. phaffii ADH2* can be assembled and integrated into a CRIS*Pi* plasmid.