Chapter 1

Introduction

1.1 Background

There is an augmenting demand for sustainable bioprocesses and production of recombinant biomolecules has prompted significant advancements in the development of microbial cell factories characterised by enhanced metabolic capabilities. Among various microorganisms used in industrial biotechnology, yeasts are widely utilised due to their rapid growth, ease of cultivation, and ability to perform post-translational modifications. A study by Karnaouri et al. (2020) highlights significant attention to the usage of non-conventional yeast alongside the conventional *Saccharomyces cerevisiae* for their unique physiological and metabolic characteristics. Among the non-conventional yeasts utilised by biotechnology industries, *Komagataella phaffii* (formerly known as *Pichia pastoris*) is extensively employed as a host for recombinant protein production, primarily due to its robust methanol-inducible promoter system, ability to sustain high cell density fermentations, and effective protein secretion capabilities (Mukherjee & Sivaprakasam, 2025; van Coeller et al., 2025). Furthermore, Wu et al. (2023) categorised *K. phaffii* as a Crabtree-negative yeast, indicating its primary reliance on respiratory metabolism rather than fermentative metabolism under aerobic conditions. These characteristics make *K. phaffii* a promising platform for engineering applications, including metabolic tweaks.

Despite its industrial relevance, Anowar & Morais (2025) suggested that metabolic pathways involved in ethanol metabolism within *K. phaffii* remain insufficiently characterised. In yeast metabolism, glucose is converted into pyruvate via glycolysis, after which pyruvate may either enter respiratory pathways or be diverted toward ethanol fermentation depending on environmental and physiological conditions (Peng et al., 2025). The ethanol fermentation pathway involves alcohol dehydrogenase

family enzymes that catalyse the interconversion between acetaldehyde and ethanol while simultaneously maintaining intracellular NADH/NAD^+ balance (Alvarado et al., 2022). Among the ADHs enzymes, alcohol dehydrogenase 2 (*ADH2*) plays an important role in ethanol-associated metabolism and cellular redox regulation. Peterson et al. (2021) report that the disruption of the *ADH2* gene could influence carbon flux distribution as well as ethanol metabolism within the cell. In industrial fermentation systems, excessive carbon flux toward ethanol formation is often considered undesirable because carbon substrates may be diverted away from biomass formation and target metabolite production (Hoeijmakers et al., 2023). Although *K. phaffii* predominantly exhibits respiratory metabolism, ethanol-associated pathways may still contribute to metabolic regulation under specific cultivation conditions, such as oxygen limitation or metabolic stress (He et al., 2024). Therefore, understanding the role of *ADH2* in *K. phaffii* metabolism is critical for understanding the carbon redistribution and informing future engineering strategies targeting *K. phaffii*'s metabolic pathways.

Recent advances in CRISPR-Cas9 have rapidly enabled precise genome engineering in various microorganisms, including non-conventional yeasts (Hasan et al., 2024). The CRISPR-Cas9 system utilizes guide RNA-directed DNA cleavage to facilitate targeted gene editing via DNA repair mechanisms (Martinez-Turillas et al., 2022). Compared to conventional genome engineering techniques, CRISPR-Cas9 offers higher specificity, efficiency, and versatility for metabolic engineering applications (Wang et al., 2023). Several studies have successfully demonstrated the application of CRISPR-Cas9 in *K. phaffii* for metabolic pathway engineering and strain improvement. In this context, generation of an *ADH2* knockout strain serves as a preliminary platform for future metabolic engineering efforts, such as the development of lactate-producing *K. phaffii* strains through the integration of heterologous *LDH* expression cassettes at a different locus. Based on these considerations, the present study aims to disrupt the ethanol fermentation pathway in *K. phaffii*

through CRISPR-Cas9-mediated deletion of the *ADH2* gene. This research is expected to provide insight and establish the feasibility of employing the CRISPR system for the deletion of the *ADH2* gene in *K. phaffii* and serves as a preliminary investigation into the effect of *ADH2* knock-out on ethanol-associated metabolism and contributing to the development of future carbon flux engineering strategies in non-conventional yeasts.

1.2 Objective

To obtain a new *K. phaffii* strain with disrupted ethanol fermentation pathways by knocking out the *ADH2* gene using a CRISPR-Cas9 genome editing system for *KpADH2* knockout, including the assembly of a homologous repair template through overlap-extension PCR, construction of a CRISPR-Cas9 plasmid containing an *ADH2*-specific sgRNA using the GoldenPiCS system, and co-transformation of both DNA components into *K. phaffii* via electroporation, thereby generating a strain in which the *KpADH2* coding sequence is deleted and replaced by the repair template cassette through homology-directed repair.

1.3 Hypothesis

There are two hypotheses that can be tested in this study.

- Null Hypothesis (H_0): CRISPR-Cas9–mediated disruption of the endogenous alcohol dehydrogenase (*ADH2*) gene and heterologous expression of the *K. phaffii* repair template do not result in a change of gene deletion in the new *K. phaffii* strain.
- Alternative Hypothesis (H_1): CRISPR-Cas9–mediated disruption of the endogenous alcohol dehydrogenase (*ADH2*) gene and heterologous expression of the *K. phaffii* repair template result in gene deletion in the new *K. phaffii* strain.