Abstract

Komagataella phaffii is a non-conventional yeast that is widely used in industrial biotechnology due to its ability to grow to high cell densities and its effectiveness as a host for genetic engineering applications. A promising strategy for metabolic engineering in *K. phaffii* involves disrupting genes associated with ethanol metabolism, such as alcohol dehydrogenase *2* (*KpADH2*). This study aimed to construct and apply a CRISPR-Cas9 system for the disruption of the *ADH2* gene in *K. phaffii*. A homologous repair template was created using overlap-extension PCR, while an *ADH2*-specific CRISPR-Cas9 plasmid was constructed using the GoldenPiCS cloning system. Both constructs were introduced into *K. phaffii* cells by electroporation and selected on kanamycin-containing media. Successful transformants were subsequently screened using colony PCR. The repair template and CRISPR-Cas9 plasmid were successfully constructed and validated. Transformation resulted in the formation of several putative colonies, indicating successful uptake of the CRISPR/Cas components. Colony PCR analysis showed that one out of three screened colonies produced the expected 2000 bp amplicon, which is 1000 bp shorter compared to the native *KpADH2*, suggesting successful removal of the *ADH2* coding region. This study demonstrates the feasibility of using CRISPR-Cas9 to target and disrupt the *KpADH2* gene, providing a basis for future research on ethanol metabolism and the development of metabolically engineered *K. phaffii* strains for industrial applications.

Keywords: *Komagataella phaffii, alcohol dehydrogenase, CRISPR-Cas9, overlap extension-PCR, Golden Gate assembly*