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Disruption of *URA3* Gene in Thermotolerant Natural Yeast by CRISPR/Cas9 Technology

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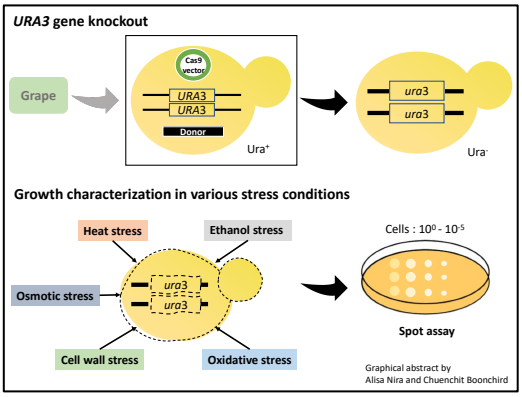
Abstract: Thermotolerant yeasts from natural sources have received much attention in industry as they are robust and can tolerate several stresses. To apply natural yeast strain as a host for production of heterologous protein, genetic marker is required for the selection of transformed cells and maintenance of recombinant plasmids. The yeast *Saccharomyces cerevisiae* strain C3253 isolated from grape in Thailand was previously characterized as thermotolerant yeast which can tolerated multiple stresses. This study aimed to construct a *ura*3 auxotrophic mutant via CRISPR/Cas9 technique which then could be transformed by yeast plasmid with a *URA*3 selectable marker. *URA*3 gene of this yeast strain was knocked out by inserting a plasmid containing a Cas9 gene under control of *GAP*1 promoter, a guide RNA expression cassette controlled by *SNR*52 promoter for specific target sites of CRISPR/Cas9, *Kan*Mx4 selectable marker, and double-stranded oligonucleotide repair template (Donor). From the observation of uracil required colonies in various selective medium as well as confirmation by DNA sequencing at the target region, the results demonstrated that *URA*3 gene in the natural yeast strain was completely knocked out. The mutant clones retained characteristic of multiple stress tolerance, i.e., heat, oxidative, osmotic, ethanol and cell wall stresses. This study suggests that CRISPR/Cas9 technique works efficiently to knockout gene of natural yeast strain for further applications.

Graphical abstract:



Keywords: Thermotolerant; *Saccharomyces cerevisiae*; CRISPR-Cas9; *URA*3; 5-FOA

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