Optimizing CRISPR/Cas9 for high-expression genome *loci* in industrial yeast strains

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The yeast *Saccharomyces cerevisiae* is one of the key cell factories for the production of bio-based chemicals, from fuels and bulk chemicals to active pharmaceuticals. Generally recognized as safe (GRAS) by the U. S. Food and Drug Administration and with a broad array of tools available at the molecular level, *S. cerevisiae* has been successfully manipulated for a wide range of applications. For large-scale fermentations, particularly in biorefineries, yeast cells must perform under harsh conditions, such as fluctuating pH and temperature, high osmotic pressure and presence of inhibitors derived from biomass hydrolysis. In this context, robust and stress-tolerant yeast chassis are required to attain high titers and product yields [1]. Industrial environments have been identified as a bioresource of yeast strains with higher robustness and fermentation performance and distinct strains have been isolated. However, such strains are more difficult to genetically manipulate than the standard laboratory strains as they are typically prototrophic, diploid and often exhibit low transformation efficiencies and lower levels of homologous recombination. In this way, efficient genetic engineering tools are required to develop effective yeast platforms. Recently, the CRISPR/Cas9 system, based on RNA-guided nuclease activity, has been employed in industrial yeasts for efficient disruption of genes and DNA insertion. This technique allows for DNA integration in single and multiple *loci*, which might be an advantage for the fine tuning of gene expression. However, genome location must be investigated for gene integration suitability by testing whether a given region supports high gene expression without affecting the microorganism's fitness. Here, we designed guide RNA's targeting conserved *Ty* elements in the yeast genome, driving the Cas9 double-strand break for heterologous DNA insertion. The gene coding for *A. niger* extracellular β-galactosidase was used as reporter gene, as high level expression has been previously achieved in *S. cerevisiae* and its activity is easily detected by the hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) [2]. The *lac*A gene, under the control of ADH1 promoter and terminator (*lac*A cassette), and flanked by homologous *Ty* sequences was used as donor DNA for homologous recombination. Moreover, a multicopy yeast expression vector carrying the *lac*A cassette and the dominant G418-resistance marker was constructed, as a comparison to multi-copy genome integrations. Selected industrial strains were transformed and expression levels assessed.

[1] Costa, C, Romani, A, Cunha, JT, Johansson, B, Domingues L. Integrated approach for selecting efficient *Saccharomyces cerevisiae* for industrial lignocellulosic fermentations: Importance of yeast chassis linked to process conditions , *Bioresource Techcnology* 227, 24-34, 2017.

[2] Oliveira, C, Guimarães, PMR, Domingues, L. Recombinant microbial systems for improved β-galactosidase production and biotechnological applications, *Biotechnology Advances*, 29(6), 600-609