GENOMIC AND EVOLUTIONARY ENGINEERING STRATEGIES IN YEASTS FOR IMPROVED BIOETHANOL PRODUCTION FROM SUGARCANE

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Sucrose is the major sugar in cane juice, a predominant feedstock for fuel-ethanol production by industrial fermentation processes. To further improve the economic and environmental sustainability of fuel ethanol production we engineered the energetics and topology of sucrose uptake and hydrolysis in the yeast *S. cerevisiae*. Our strategy is based on the knowledge that two different ways by which yeasts can utilize sucrose are known: this sugar can be hydrolyzed by the extracellular enzyme invertase, producing glucose and fructose which are transported into the cells and metabolized, or the disaccharide can be actively transported into the cells by H^+-symport and hydrolyzed intracellularly. Indeed, early work on the *SUC* genes of *S. cerevisiae* already showed that these genes encode for the two forms of invertase, including an intracellular enzyme, and the secreted glycosylated form of invertase. However, extracellular sucrose hydrolysis allows growth of other microorganisms, including contaminant yeasts and bacteria lacking invertase, while extracellular production of fructose also imposes several problems to the industrial process due to slower fructose utilization by *S. cerevisiae* cells, which may result in residual sugar at the end of the cultivation with consequent losses in productivity.

Due to the energetics of active sugar transport, it is expected that cells that exclusively transport sucrose into the cells will exhibit a higher ethanol yield on sucrose than the wild-type phenotype, in order to compensate the ATP expenditure of extruding the H^+ originated from the sucrose-H^+ symport activity. Thus, to improve sucrose fermentation by yeasts, we have modified the *SUC2* gene encoding invertase using genomic engineering (producing stable chromosomal modifications) so that now the gene lacks the N-terminal signal peptide responsible for secretion of the enzyme, overexpressing exclusively the intracellular form of invertase (pADH1::*iSUC2*). Consequently, in this modified *iSUC2* strain sucrose needs to be actively transported into the cells for fermentation, without glucose and fructose release into the medium. Indeed, in the *iSUC2* strain the active sucrose-H^+ symport activity and intracellular invertase levels were 4- and 22-fold higher, respectively, when compared to the wild-type strain.

An evolutionary engineering strategy aimed to improve the performance of the *iSUC2* yeast cells allowed obtaining, after 30-40 generations in anaerobic sucrose-limited chemostat cultures, an evolved strain that showed improved fermentation performance. The evolved strain exhibited a 12-fold increased sucrose-H^+ symport activity, and a 17-fold lower residual substrate concentration in the chemostat, while the invertase levels were unaffected. This evolved *iSUC2* strain showed an 8-11% higher ethanol yield on sucrose, both during chemostat cultivations or in batch fermentations at high cell densities (simulating the industrial production of fuel ethanol), as well as 50% higher rates of ethanol and CO_2 production. Transcriptome analysis of this *iSUC2* evolved yeast strain revealed overexpression of several *HXT* sugar transporters, as well as many *MAL*-regulated genes, including the high-affinity sucrose-H^+ symporter encoded by the *AGT1* gene. Further analysis of this *iSUC2* evolved strain revealed indeed a duplication of this gene, and when the two copies of *AGT1* were deleted from the genome of the evolved strain, the yeast cells lost their higher sucrose fermentation performance, with ethanol yields and production rates similar to those found in the original wild-type yeast strain.

We finally introduced this *iSUC2* strategy (including the overexpression of the *AGT1* gene) not only into diploid yeast strains, but also into a fuel-ethanol industrial yeast strain currently used in large scale by the Brazilian sugarcane mills. Our genome analysis of several dominant and efficient fuel-ethanol yeast strains revealed bona-fide diploid genomes containing important gene copy number variations probably adaptive for the industrial environment. These fuel-ethanol yeast strains have only *SUC2* in their genomes, and a pADH1::*iSUC2* + pTDH3::*AGT1* derivative industrial strain that not only lacks extracellular invertase, but overexpresses the intracellular invertase and the high-affinity sucrose-H^+ symporter, fermented high sucrose concentrations efficiently in the same time used by the parental

industrial strain, without glucose or fructose release into the medium. It is important to note that the present strategy to produce a more efficient strain for fuel-ethanol production relies in the modification of the known metabolic pathways and expression of the *S. cerevisiae* own genes, a method also called “self-cloning” that does not have any restriction for use or commercialization in the industrial sector by current regulatory policies. The fermentation performance and dominance of this industrial modified yeast strain is currently being tested in the industrial environment of a sugarcane mill, where we expect a profound impact on the overall process performance. A yeast strain that ferments sucrose directly will certainly contribute to a more efficient bioethanol production also from lignocellulose biomass, as there will be no glucose or fructose to compete with xilose uptake and fermentation by the yeast cells during sucrose/xylose co-fermentations. Thus, the current work constitutes a significant and innovative report on the improvement of the fermentation performance of the yeast *S. cerevisiae*, which will help to further improve the economical and environmental sustainability of sugarcane-based bioethanol production.

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