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Improving conversion yield of fermentable sugars into fuel ethanol in 1st generation yeast-based production processes

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Current fuel ethanol production using yeasts and starch or sucrose-based feedstocks is referred to as 1st generation (1G) ethanol production. These processes are characterized by the high contribution of sugar prices to the final production costs, by high production volumes, and by low profit margins. In this context, small improvements in the ethanol yield on sugars have a large impact on process economy. Three types of strategies used to achieve this goal are discussed: engineering free-energy conservation, engineering redox-metabolism, and decreasing sugar losses in the process. Whereas the two former strategies lead to decreased biomass and/or glycerol formation, the latter requires increased process and/or yeast robustness.

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Introduction

90 billion liters of fuel ethanol are currently produced worldwide (Renewable Fuels Association; URL: www.ethanolrfa.org) using almost exclusively starch or sucrose containing feedstocks. The hexose sugars released from for instance corn starch (by industrial hydrolytic enzymes) or sugar-cane derived sucrose (by yeast invertase) can directly be fermented to ethanol by yeast. These processes are referred to as 1st generation (1G) fuel ethanol production. With a high contribution (up to 70%) of the feedstock to the final production cost [1], high production volumes and small profit margins, the overall conversion yield of the raw material into ethanol is crucial for the process economy. This review focuses specifically on recent scientific advances with the potential to improve the ethanol yield on sugar. Strategies that aim at increasing ethanol titers, such as very high gravity fermentation, resulting in decreased distillation costs, decreased contamination risks, and decreased vinasse production [2], are beyond the scope of this review.

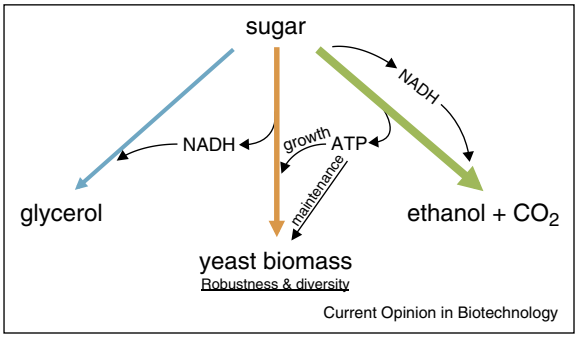
Conversion of 1 mol of hexose sugar into 2 mol ethanol and 2 mol CO_2 is a redox-neutral conversion (Figure 1). This makes the maximum theoretical yield 0.51 g ethanol per g hexose sugar. Industrial ethanol production operates at >90% of this theoretical yield [3]. Yeast biomass and glycerol are the two main by-products of ethanol production, besides the unavoidable production of CO_2. Under anaerobic conditions alcoholic fermentation of sugars is the sole pathway in yeast that provides energy in the form of ATP for cellular maintenance and, if sufficient ATP is available, for growth. When ATP is used for growth, yeast biomass and accompanying glycerol (see below) are formed at the expense of feedstock that is not converted to ethanol (Figure 1). Any reduction in yeast biomass production and glycerol formation will result in increased ethanol yields.

Industrial ethanol processes are often carried out without complete asepsis. Growth of contaminating microorganisms can divert sugar away from ethanol formation or even result in incomplete fermentations due to accumulation of toxic compounds. Use of robust yeast strains that can operate at high ethanol concentrations and at decreased pH creates a selective advantage over potential contaminants and decreases these losses.

Engineering free energy conservation to increase ethanol yield

Growth of yeast and the accompanying glycerol formation diverts carbon away from ethanol production. The extent of this growth is dependent on the availability of energy in the form of ATP (Figure 1). If the ATP yield on sugar is decreased, this increases the ethanol yield on sugar in two ways [4]. Firstly, more sugar has to be converted solely to ethanol and CO_2 to provide the same amount of ATP for cellular maintenance. Secondly, in a strain with a decreased ATP yield on sugar, but with identical biomass yield on ATP, an increased fraction of the sugar is converted to ethanol, simultaneously decreasing the biomass and glycerol yields.

Figure 1



Schematic representation of the distribution of sugar for ethanol production, formation of yeast biomass, and formation of glycerol as a by-product. To achieve a high ethanol yield on sugar, the robustness of the process and yeast strains are essential.

Replacing the Embden–Meyerhof glycolysis, which yields 2 ATP per hexose, by a heterologous Entner–Doudoroff pathway that yields 1 ATP per hexose would decrease the ATP yield on sugar. To investigate this possibility, Benisch and Boles [5•] constructed a yeast strain containing 6-phosphogluconate dehydratase and 2-keto-3-deoxygluconate-6-phosphate (KDPG) aldolase from *Escherichia coli*. High activities were shown for KDPG-aldolase. However, activities of the heterologous 6-phosphogluconate dehydratase were insufficient for functional replacement of the Embden–Meyerhof glycolysis by the Entner–Doudoroff route, which was attributed to poor assembly of the [4Fe–4S] iron–sulfur cluster of the 6-phosphogluconate dehydratase in yeast. These findings illustrate that functional expression of bacterial proteins containing iron–sulfur clusters remains a challenge in yeast metabolic engineering [5•,6].

Engineering the stoichiometry of sugar transport provides another opportunity to decrease the ATP-yield on sugar. Wild type *Saccharomyces cerevisiae* strains hydrolyze sucrose extracellularly and use facilitated diffusion to take up glucose and fructose. When this mechanism is replaced by sucrose uptake via proton symport and intracellular hydrolysis, the ATP requirement for subsequent proton extrusion decreases the anaerobic ATP yield on sucrose from 4 to 3. Requiring a combination of metabolic and evolutionary engineering, this strategy resulted in an 11% increase of the ethanol yield on sucrose [7•] (Table 1). This same strategy can in theory be applied to replace the facilitated diffusion of the hexose sugars with transport via proton-symport, resulting in a 50% decrease in the ATP yield from 2 to 1 mol per mol of hexose. That this strategy not necessarily requires heterologous transporters, was shown by the characterization of the fructose/H^+ symporter Fsy1 from a wine strain of *S. cerevisiae* [8,9].

Whereas the abovementioned strategies all rely on changing the stoichiometry of ATP formation in sugar metabolism, other strategies apply non-stoichiometric ATP drains by intervening in ATP or H^+ homeostasis. A classic example of this strategy is introduction of ATP-hydrolyzing futile cycles in yeast through the deregulation of some gluconeogenic enzymes [10]. A recent attempt to increase ATP hydrolysis, thereby potentially decreasing growth and increasing alcoholic fermentation, encompassed the overexpression of ATPase [11]. Further studies are required to quantify the impact on the ethanol yield under industrially relevant conditions. In another study, the authors claim that overexpression of alkaline phosphatase Pho8 increased the ethanol yield on sugar by up to 13%, despite a small impact on intracellular concentrations of ATP [12]. However, the challenge with the introduction of such non-stoichiometric ATP drains, especially for industrial implementation, is in the fine tuning between the positive impact and decreased cellular robustness.

Decreasing formation of glycerol as a by-product to increase the ethanol yield

Glycerol is the 3rd major by-product of alcoholic fermentation after co-production of CO_2 and yeast biomass. It is estimated that in industrial fermentations approximately 4% of the sugar feedstock ends up as glycerol [13]. In anaerobic yeast fermentations, formation of glycerol is essential to re-oxidize surplus NADH resulting from growth on sugars. Additionally, glycerol is the main compatible solute in yeast, produced in response to the high osmotic pressure that can occur in some process configurations. A first approach to minimize the formation of

Table 1

Selected strategies to increase ethanol yield on sugar in first generation fuel ethanol production

	Genetic strategies				Process strategies
	Breeding/biodiversity	Recombinant DNA	Evolution	Shuffling	
Desired traits of different strains into one strain	[25•,53]				
Decrease glycerol	[14•,15]	[17,20,21•,24•]			
Decrease free-energy conservation		[5•,7•,11,12]	[7•]		
Increase stress tolerance in yeast		[35,46,34]	[36]	[32,33]	
Decrease contamination					[27,30,31•]