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Commentary

COMMENTARY

Ethanol yield calculations in biorefineries

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One sentence summary: The ethanol yield on sugar in sugarcane biorefineries is a more complex process parameter to be determined than ethanol yields calculated from laboratory experiments and has probably been overestimated.

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ABSTRACT

The ethanol yield on sugar during alcoholic fermentation allows for diverse interpretation in academia and industry. There are several different ways to calculate this parameter, which is the most important one in this industrial bioprocess and the one that should be maximized, as reported by Pereira, Rodrigues, Sonego, Cruz and Badino (A new methodology to calculate the ethanol fermentation efficiency at bench and industrial scales. *Ind Eng Chem Res* 2018; 57: 16182–91). On the one hand, the various methods currently employed in industry provide dissimilar results, and recent evidence shows that yield has been consistently overestimated in Brazilian sugarcane biorefineries. On the other hand, in academia, researchers often lack information on all the intricate aspects involved in calculating the ethanol yield in industry. Here, we comment on these two aspects, using fuel ethanol production from sugarcane in Brazilian biorefineries as an example, and taking the work of Pereira, Rodrigues, Sonego, Cruz and Badino (A new methodology to calculate the ethanol fermentation efficiency at bench and industrial scales. *Ind Eng Chem Res* 2018; 57: 16182–91.) as a starting point. Our work is an attempt to demystify some common beliefs and to foster closer interaction between academic and industrial professionals from the fermentation sector. Pereira, Rodrigues, Sonego, Cruz and Badino (A new methodology to calculate the ethanol fermentation efficiency at bench and industrial scales. *Ind Eng Chem Res* 2018; 57: 16182–91).

Keywords: fuel ethanol production; alcoholic fermentation; industrial biotechnology; ethanol yield; sugarcane biorefinery

The ethanol yield on sugar during yeast fermentation seems to be a straightforward parameter to determine, intuitive to understand and simple to calculate. Academic researchers, when considering laboratory cultivations, would immediately conceive this determination in one of the following ways. In a batch cultivation, carried out either in a bioreactor, in a shake-flask or even using any of the several currently available milli- or micro-scale systems, one needs to determine the

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initial and final volumes and concentrations of ethanol and sugars present in the cultivation medium. There are several analytical options for this purpose. The ethanol concentration can be measured using distillation and a colorimetric determination (Williams and Darwin Reese 1950; Verduyn, van Dijken and Scheffers 1984), enzymatic assays (de Marcos *et al*. 1997; Caudy 2017), chromatographic methods—either gas chromatography (GC, Stackler and Christensen 1974; Playne 1985) or high-performance liquid chromatography (HPLC, Bonn and Bobleter 1984)—, or even near- (NIR, Liebmann, Friedl and Varmuza 2010; Nascimento *et al*. 2017; Krämer and King 2019) or mid-infrared (MIR, Kansiz *et al*. 2005; Debebe, Redi-Abshiro and Chandravanshi 2017; Rodrigues *et al*. 2018) spectroscopy. It can also be determined indirectly using a densitometer after distilling ethanol from the sample, as there is a correlation between the composition of ethanol/water mixtures and their densities within the concentration range encountered in industry.

There are also various analytical methods to determine the concentration of sugars. Considering the most common mono- and di-saccharides present in sugarcane biorefineries, such as glucose, fructose and sucrose, enzymatic assays (Caudy 2017), HPLC (Palmer and Brandes 1974; Palmer 1975; Bonn and Bobleter 1984) or methods that can quantify the reducing sugars released after the hydrolysis of sucrose, can be employed (Rodrigues *et al*. 2018).

A simple formula can be applied to calculate the ethanol yield ($Y_{ETH/S}$) from experimental data (Equation 1).

$$Y_{ETH/S} = (ETH_{final} - ETH_{initial})/(TRS_{initial} - TRS_{final}), \quad (1)$$

where ETH and TRS are the total masses or moles of ethanol and total reducing sugars (free glucose and fructose plus the same monosaccharides released from sucrose hydrolysis), respectively.

The mass or molar values above (ETH or TRS) are calculated by multiplying the measured concentration (in mass/volume or moles/volume) by the cultivation volume at the time the corresponding sample was taken. When volume remains constant during the entire cultivation, the measured concentration can be directly used in Equation 1, instead of the masses or moles.

In the sugarcane ethanol industry in Brazil, the ethanol concentration in the fermented *must* is routinely determined by distilling the sample and measuring the density of the distillate using a densitometer (IUPAC 1968; Zago *et al*. 1989). For calculating ethanol yield, more reliable measurements are needed, such as those based on chromatography. On the other hand, sugar content in the *must* before fermentation is determined using two different methods: (1) indirectly estimating the degrees Brix, either using a densitometer (ICUMSA—https://www.icumsa.org/index.php?id=1670) or a refractometer, and (2) directly measuring the concentration of TRS. While the latter value—being more precise than the former—is the one used to calculate ethanol yields, analysis via densitometry is easier and more suitable for process monitoring. A constant density value indicates that the fermentation has ended. Additionally, the end of the fermentation can also be identified by monitoring the process water consumption; when cooling water is no longer required, this is an indication that fermentation has ceased.

The yield in Equation 1 can be considered a *global process parameter*, which does not capture the behavior of the yeast cells during the different phases of a typical batch cultivation (lag, log, deceleration and stationary) and, frequently, the initial concentration of ethanol and the final concentration of sugars are equal to zero. Thus, it is a simple parameter to calculate. However, we would like to emphasize two important aspects of this parameter, calculated as mentioned above: (1) it will be very much dependent on the accuracy of the analytical determinations, since only two timepoints are used, and eventual errors in the sampling procedure and/or in the analytical determination will affect the calculation significantly; and (2) it is a *process parameter* and not a *physiological parameter*, due to the reasons mentioned above. Also, it should be noted that in industry, fermentation is sometimes finished before all sugars have been consumed (possibly because of unknown issues regarding yeast performance). Despite this, the ethanol yield is calculated considering full exhaustion of sugars, because this is the yield that matters in the industry. From Equation 1, one can see that this will lead to a lower yield. Very rarely all sugars are consumed, and yeast might continue to produce ethanol from storage carbohydrates, such as trehalose and glycogen, which could lead to an overestimation of the yield (Basso *et al*. 2008). However, under normal processing conditions, this 'endogenous fermentation' can be neglected.

Eventually, in the case of significant ethanol evaporation during lab cultivations, a correction factor can be applied to obtain a more realistic value. This correction factor could be calculated, e.g. by incubating a mixture of water and ethanol under the exact same conditions used for the yeast cultivation and determining how much ethanol evaporates from the system (Madeira-Jr and Gombert 2018). In Brazilian sugarcane biorefineries, it has been estimated that less than 1% of the ethanol leaves the fermenter with the off-gas, and almost all companies use scrubbers to recover it using water. However, it is difficult to include this recovered ethanol in yield calculations for individual vats, because the scrubbers are not attached to individual fermentors. There is normally only one gas scrubber for washing the CO_2 streams from all vats of the fermentation unit.

Another way to calculate the ethanol yield during a batch cultivation is to consider only the exponential (or LOG) phase of yeast growth. In this case, samples can be taken at various time points during the LOG phase for the determination of the ethanol and sugar concentrations, using the methodologies listed above. With these data, it is possible to construct a graph relating ETH with TRS, which will display data points that follow a linear relationship, because ethanol is a primary (or catabolic) metabolite, whose formation is strictly coupled to cell growth, generating ATP under anaerobic conditions. By performing a linear regression with these data points only during the LOG phase, it is possible to calculate the ethanol yield on sugars, which will correspond to the absolute value of the slope. Important aspects of this option are: (1) the calculation will be less sensitive to an analytical error in the concentration of ethanol and/or sugars in one specific data point, since several data points are used in the calculation (the more, the better); (2) this is truly a *physiological parameter* and not a *process parameter*.

In the case of a continuous cultivation, typically carried out in a bioreactor (at bench or industrial scale), the ethanol yield can be calculated using the same analytical methods and the following formula, in case of a steady-state cultivation (Equation 2):

$$Y_{ETH/S} = (F_{out} \cdot [ETH]_{out} - F_{in} \cdot [ETH]_{in})/(F_{in} \cdot [TRS]_{in} - F_{out} \cdot [TRS]_{out}), \quad (2)$$

where F is a volumetric flow rate (in volume/time), [ETH] and [TRS] are the mass or molar concentrations (in mass/volume or moles/volume) of ethanol and TRS, respectively; 'out' and 'in' are

subscripts that identify the outlet and the inlet streams, respectively.

The concentration values of ethanol and TRS in the cultivation broth will be unique only in the case of a perfectly mixed system, with no concentration gradients, which is commonly achieved in small-scale laboratory cultivations. In this case, the concentration inside the cultivation vessel and in the outlet medium will be the same, and eventually, measurements can be performed directly by taking a sample from the outlet stream, if this is simpler. The yield in Equation 2 can be considered both a *process parameter* and a *physiological parameter*. In other words, both parameters are the same in a chemostat.

Finally, in the case of transient continuous cultivations or in the case of a fed-batch cultivation, proper mass balances can be applied and from these the instantaneous ethanol yield can be calculated, provided the remaining variables and parameters are known (Equations 3 and 4):

$$d(V \cdot [TRS])/dt = F_{in} \cdot [TRS]_{in} - F_{out} \cdot [TRS]_{out} - (\mu/Y_{X/S}) \cdot [X] \cdot V, \quad (3)$$

$$d(V \cdot [ETH])/dt = F_{in} \cdot [ETH]_{in} - F_{out} \cdot [ETH]_{out} + (Y_{ETH/S} \cdot \mu/Y_{X/S}) \cdot [X] \cdot V, \quad (4)$$

where V is the cultivation volume, [TRS] is the TRS concentration (in mass/volume), F is the volumetric rate of medium (volume/time), μ is the specific growth rate (in time^{-1}), [X] is the cell concentration (in dry cell mass/volume), [ETH] is the ethanol concentration (in mass/volume), $Y_{X/S}$ is the biomass yield on sugar (in dry cell mass/mass sugar), 'in' is the subscript for inlet and 'out' is the subscript for outlet.

For fed-batch and transient continuous cultivations, it is also possible to determine a global ethanol yield on sugar (similarly to what was discussed above for batch cultivation), by simply dividing the total amount of ethanol produced in the process ($V_{final} \cdot [ETH]_{final} — V_{initial} \cdot [ETH]_{initial}$) by the total amount of sugar utilized ($V_{initial} \cdot [TRS]_{initial} — V_{final} \cdot [TRS]_{final}$), which, in fed-batch cultivation refers to the total amount of sugar fed to the reactor. Such a global yield can be considered a *process parameter*, whereas the instantaneous yield in Equation 4 is rather a *physiological parameter*. If the physiological parameter does not change significantly along the cultivation, it will be close to the global yield.

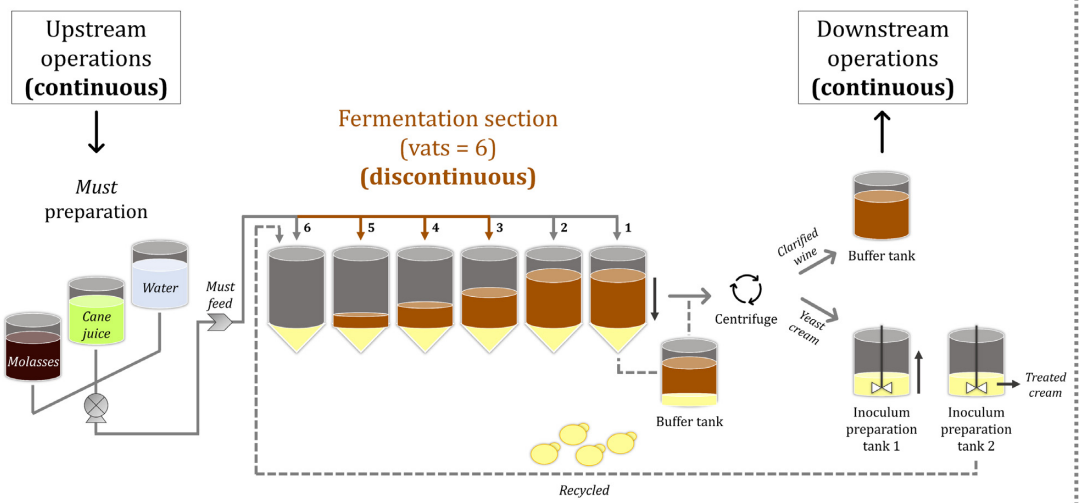
The situation in industry is dramatically different from the one in laboratories, but we believe this has been taken for granted by the academic community, including the authors of this commentary. Among other aspects, the real ethanol yield on sugars in industry serves as a benchmark for metabolic engineering and other strain improvement strategies aiming at increasing it. The ethanol yield on sugar is the most important parameter in processes where the substrate represents the major cost of the whole industrial operation. In sugarcane biorefineries, sugar accounts for ~70% of all OPerational EXpenses (OPEX; Gombert and van Maris 2015), which include at least: sugarcane crushing, broth treatment and preparation, fermentation, centrifugation, acid treatment of the yeast cream for cell recycling, distillation and vinasse disposal, laboratory analyses, wages and salaries. Considering the three main parameters used by engineers to evaluate industrial processes, namely Titre, Rate (or Productivity) and Yield (making up the TRY acronym), yield needs to be prioritized in this kind of process, at the expense of the other two parameters. Thus, it is of utmost importance that the yield is calculated in the most accurate manner, which brings us to the main point of this commentary: 'How is the ethanol yield calculated in sugarcane biorefineries?'

First, it needs to be clarified that in a sugarcane-based biorefinery most of the operations—both upstream and downstream of the fermentation section—run in continuous mode. Fermentation, typically carried out as a fed-batch process, contrasts with the remaining operations (Fig. 1). Some upstream continuous operations, such as *must* preparation, are then connected to the fermentation step, which consists of several fermenters, typically 6–10, where fermentations occur in parallel, asynchronously. The *must* is prepared using an inline continuous mixture of streams of molasses and sugarcane juice or water, to target a TRS concentration around 160 g/L. The inline mixing system immediately upstream of fermentation avoids the necessity for holding tanks with prepared medium that would otherwise facilitate contamination; molasses from table sugar production is kept in holding tanks at 500–600 g/L TRS, an unfavorable condition for most microbes. The *must* is fed directly into one fermentation vat over ~4 h, which initially contains the acid-treated yeast cream (inoculum). After one fermenter has been fed to its maximum working volume, the feed medium starts to be added to another fermenter and so on, but depending on the industry's setup, there might be two or more fermenters being filled in parallel (see a typical scheduling scheme in Fig. 1B). When a fermenter is ready for discharging (end of fermentation), its content (*wine*) is first transferred to a buffer tank, from which it is continuously fed to centrifuges (that operate in continuous mode). While the supernatant (one of the streams leaving the centrifuge) is transferred to another buffer tank, to link with downstream continuous distillation operations, the yeast cream is sent to an acid treatment tank, from which inocula for the different fermenters are prepared.

This structure *per se* already poses a challenge in yield determination, and begets a question: 'Where, when, and how often the concentrations of ethanol and sugars should be determined'? As presented and discussed in detail by Pereira *et al.* (2018), it is possible to determine an average yield, e.g. for an entire month of operation, by dividing the total amount of ethanol produced in the factory by the total amount of sugar that entered the facility. This value can be quite accurate, but it does not provide any information on the performance of individual fermentations (which typically last around 8 h). This approach is rather process monitoring *a posteriori* than real time monitoring, which could be useful for process control actions to be put in place to correct for eventual problems. For instance, a low yield during (or immediately after) a run could be related to a high contamination level (factories also monitor bacterial rod counts), to the presence of an invading yeast strain with an inherent lower ethanol yield, to improper medium preparation or to inadequate temperature control, among other possibilities. Once the problem has been identified, actions can be taken to avoid an underperformance in subsequent runs. In general, the earlier a problem (in this case a low ethanol yield) is identified, the sooner a process can return to the desired operating conditions.

As Pereira *et al.* (2018) describe in their work, there are at least four approaches commonly employed in the fuel ethanol industry from sugarcane for the determination of the ethanol yield. Some deliver yields over a longer time range and others allow for the evaluation of a more batch-wise ethanol yield, which is useful to rectify problems. Among these, there is an approach — widely used in Brazilian distilleries — based on the consideration that the amount of ethanol produced is calculated from

Panel A



Panel B

Time (h)	Fermentation section						Pre/post fermentation section	
	Vat 1	Vat 2	Vat 3	Vat 4	Vat 5	Vat 6	IPT* 1	IPT* 2
1	Transfer	Centr.	On Hold	Feeding	Feeding	Feeding	Transfer	Filling
2	Feeding	CIP	Centr.	On Hold	Feeding	Feeding	Filling	Treatment
3	Feeding	Transfer	Centr.	On Hold	Feeding	Feeding	Filling	Transfer
4	Feeding	Feeding	CIP	Centr.	On Hold	Feeding	Treatment	Filling
5	Feeding	Feeding	Transfer	Centr.	On Hold	Feeding	Transfer	Filling
6	Feeding	Feeding	Feeding	CIP	Centr.	On Hold	Filling	Treatment
7	Feeding	Feeding	Feeding	Transfer	Centr.	On Hold	Filling	Transfer
8	On Hold	Feeding	Feeding	Feeding	CIP	Centr.	Treatment	Filling
9	On Hold	Feeding	Feeding	Feeding	Transfer	Centr.	Transfer	Filling
10	Centr.	On Hold	Feeding	Feeding	Feeding	CIP	Filling	Treatment
11	Centr.	On Hold	Feeding	Feeding	Feeding	Transfer	Filling	Transfer
12	CIP	Centr.	On Hold	Feeding	Feeding	Feeding	Treatment	Filling
13	Transfer	Centr.	On Hold	Feeding	Feeding	Feeding	Transfer	Filling
14	Feeding	CIP	Centr.	On Hold	Feeding	Feeding	Filling	Treatment
15	Feeding	Transfer	Centr.	On Hold	Feeding	Feeding	Filling	Transfer
16	Feeding	Feeding	CIP	Centr.	On Hold	Feeding	Treatment	Filling
17	Feeding	Feeding	Transfer	Centr.	On Hold	Feeding	Transfer	Filling
18	Feeding	Feeding	Feeding	CIP	Centr.	On Hold	Filling	Treatment
19	Feeding	Feeding	Feeding	Transfer	Centr.	On Hold	Filling	Transfer
20	On Hold	Feeding	Feeding	Feeding	CIP	Centr.	Treatment	Filling
21	On Hold	Feeding	Feeding	Feeding	Transfer	Centr.	Transfer	Filling
22	Centr.	On Hold	Feeding	Feeding	Feeding	CIP	Filling	Treatment
23	Centr.	On Hold	Feeding	Feeding	Feeding	Transfer	Filling	Transfer
24	CIP	Centr.	On Hold	Feeding	Feeding	Feeding	Treatment	Filling

*IPT: Inoculum Preparation Tank.

Figure 1. Interplay among the continuous upstream operations, the discontinuous fermentation units and the continuous downstream operations in a typical sugarcane biorefinery in Brazil (panel A). The situation depicted in panel A corresponds to the schedule presented in panel B at 11 h of operation, where one full day of operation is displayed. For the fermentation section: *CIP*: Clean in Place. *Transfer*: Fermenter is inoculated with yeast cream (vat 6). *Feeding*: Fermenter is fed with *must* (vats 3, 4 and 5). *On Hold*: Fermenter is fully filled, and fermentation is finishing (vat 2). *Centr.*: Fermenter is discharging to centrifugation (vat 1). For the pre/post fermentation section: *Treatment*: Yeast cream treatment with sulfuric acid (and eventually also with antimicrobials) for cell recycling. *Filling*: Receiving the yeast from the centrifuge (IPT 1). *Transfer*: Inoculation of the fermenter with yeast cream (IPT 2).

a carbon balance and by subtracting all the remaining (measured) products of yeast metabolism (Methodology 2 in Pereira *et al.* 2018). This approach seems to overestimate the real ethanol yield and should be discontinued, although it can be useful to identify problems that did not become evident from other analyses. The main reason for this overestimation is the fact that the methodology does not discount the volume of yeast cells when determining the ethanol produced in the fermentation process. Furthermore, it is not based on measurements of the raw material, making it sensitive to feedstock variability. Pereira *et al.* (2018) propose a new method that allows for the determination of an ethanol yield based on mass balances and seems to be more reliable and feasible to be widely implemented. As the exact mass of ethanol in the fermentation vat is calculated by multiplying the concentration of ethanol by the volume of the cell-free broth, the approach is based on considering the volume occupied by the yeast cells, discounting them from the *inoculum* and *wine* volumes in the determination of the ethanol yield. The

mass of ethanol produced in a fermentation is thus calculated as follows (equation 5):

$$ETH_{produced} = ETH_{final} - ETH_{initial} = V_{L-W} \cdot [ETH]_{wine} - V_{L-I} \cdot [ETH]_{inoculum}, \quad (5)$$

where V_{L-W} and V_{L-I} are the volumes of the fermented wine and of the inoculum, both without cells. $[ETH]_{wine}$ and $[ETH]_{inoculum}$ are the concentrations of ethanol in the fermented wine and in the inoculum, respectively. The two latter variables can be measured, whereas the two former ones can be calculated as follows (Equations 6 and 7):

$$V_{L-W} = V_W \cdot [1 - (C_{Y-M-W} \cdot \rho_W)/(\rho_Y \cdot \sigma)], \quad (6)$$

$$V_{L-I} = V_I \cdot [1 - (C_{Y-M-I} \cdot \rho_I)/(\rho_Y \cdot \sigma)], \quad (7)$$

where V_W and V_I are the volumes of the wine and of the inoculum, respectively (including cells); C_{Y-M-W} and C_{Y-M-I} are the dry cell mass concentrations in the wine and in the inoculum, respectively; ρ_Y is the yeast cell density (in mass/volume); σ is the dry mass content of the yeast cell (in dry cell mass/total cell mass); ρ_W and ρ_I are the densities (in mass/volume) of the wine and of the inoculum, respectively. All these variables can be measured and some of them, such as ρ_Y, σ, ρ_W and ρ_I, need not be measured on a routine basis.

Another example that clearly shows the importance of taking the volume of solids into account, when calculating yields, was reported by Kristensen *et al.* (2009). At 30% initial solids content, excluding the solid fraction in the calculation led to a 36% overestimation of the hydrolysis yield of hydrothermally pre-treated wheat straw. Instead of a laborious and precise correction factor for each substrate that considers the specific gravity of the aqueous phase and the mass of solids, they proposed an approximation method. By taking a representative slurry sample, and by diluting it ten times (on a mass basis), the error caused by the solids content decreased to a maximum of 3–5% (Kristensen *et al.* 2009).

For a more accurate calculation of the ethanol yield on sugars, fermentation companies need to invest in a better design of the sampling schemes, in the necessary analytical structure, and hire more employees to perform these analyses. However, we believe that this investment is worthwhile, since the current situation, in which companies cannot correlate the ethanol yield with any process variables, simply because they cannot properly calculate the referred yield, leads to a less efficient process, resulting in financial and environmental losses, the magnitudes of which are currently unknown.

Lastly, if the industrial ethanol yield on sugar is in reality around 85% of the theoretical maximum value (0.511 g of ethanol per g of hexose-equivalent), and not around 90% of this maximum threshold, as imagined hitherto (Della-Bianca *et al.* 2013; Pereira *et al.* 2018), there is more incentive for academic and industrial researchers to pursue, design, implement and test metabolic engineering strategies or other genetic improvement approaches, aiming at creating yeast strains with higher ethanol yields. In addition, it is important to mention that in the industrial process, the maintenance energy of the cells is expected to be much higher than in laboratory cultivation. In industry, cells are constantly exposed to high ethanol titres during the whole fermentation period, even in the beginning of each fermentation cycle (Basso *et al.* 2008; Della-Bianca *et al.* 2013). There are also serious nutritional limitations and the prevalence of toxic compounds in real-world substrates (Lino, Basso and Sommer 2018; Walker and Basso 2020). Moreover, the peculiar acid treatment stress imposed on yeast cells is of great relevance (Della-Bianca *et al.* 2014). With no doubt, all these conditions increase the maintenance energy of cells during ethanol fermentation.

In a 35 billion litres per annum fuel ethanol production scenario, as is the case with the Brazilian sugarcane-based industry (Jacobus *et al.* 2021), a 1% increase in the ethanol yield represents 350 million litres additional ethanol produced from the same area of planted sugarcane. It also represents 8.2×10^9 MJ of energy, which is equivalent to 0.2 mega tonnes of oil equivalent (Mtoe). Thus, *ca.* 0.1 million tonnes less CO_2 is released in the atmosphere when this ethanol is used in flex-fuel cars, decreasing the contribution from the transportation fuel towards global climate change.

Other (less scientific) aspects of this reality are the following. There is a lack of R&D activity in most of the ~420 Brazilian sugarcane-based biorefineries currently in operation, mainly in those industrial units that are not part of a consolidated industrial group. Even for some more sophisticated types of analyses, biorefineries usually rely on a handful of consulting companies, which keep the knowledge around this process for themselves. This is different from the North American reality, where substantial R&D activities take place in-house, in corn- or even cellulose-based fuel ethanol producing biorefineries (Ethanol Producer Magazine 2008; TheDigest 2017; POET 2019; Cargill 2020). One of the key motivations for the work published by Pereira *et al.* (2018) was to identify the so-called 'undetermined losses' observed in many industrial units. At the end of every sugarcane crushing period, factories analyse how much sugar (in sugarcane) entered the biorefinery and how much table sugar and ethanol were produced. And mass balances would not close for several reasons. There is always ethanol lost along the way and it has been difficult to identify the causes for this disappearance. In the industrial operations responsible for ethanol production (fermentation and distillation), Pereira *et al.* (2018) have tried to show that the fermentation yield has been overestimated in the past decades, which could be a plausible explanation for these 'undetermined losses'.

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