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Document Abstract

Second generation sugars, such as Clariant's wheat straw hydrolysates (WSH), contain a mixture of sugars such as glucose and xylose, associated with impurities, among which are several fermentation inhibitors. With the aim of developing an isobutene (IBN) producing bacterial strain adapted to WSH, GBE conducted an extensive survey of the performance of its current strains on this type of 2G sugars. The major parameters that were investigated were (i) growth (ii) production of compounds of interest including isobutene (iii) sugar consumption. First generation sugars and synthetic mixtures mimicking the sugar composition of WSH were tested in parallel.

WSH appeared to have some inhibitory impact on growth, depending on the experimental conditions, and on isobutene production compared to synthetic mixtures. The inhibitory effect seems to be mostly due to impurities, as shown by the good performances of runs conducted with synthetic mixtures. Glucose and xylose where both consumed efficiently. However, at this stage, one cannot exclude the building up of one of the sugars on the long term (several days).

In order to overcome the inhibitory effect, strain adaptation was initiated, and within a few weeks, a large part of the inhibitory effect was alleviated successfully. This study will have to be pursued over the next months, in order to achieve perfect fitting. Because of the public (PU) nature of this deliverable, details such as WSH composition, strain genotype and other parameters will not be disclosed in this document.

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Abbreviations

IBN: Isobutene

WSH: Clariant's Wheat Straw Hydrolysate

DCW: Dry Cell Weight

gDCW/L: Grams of Dry Cell Weight per liter

AU: Arbitrary Units

TU: Time unit

1. INTRODUCTION

In the entire field of industrial biology, 2G feedstocks, such as Lignocellulosic (LC) sugars represent an attractive alternative to corn syrup and other 1G sugars. Indeed, they can be made from widespread resources, and do not titrate or compete with food or feed production. However, the technologies currently used to produce LC sugars deliver complex syrups, which impact the fermentation performances and the purification step for the desired molecule.

First, LC sugars cannot be dissociated from several impurities, including known inhibitors of fermentation (notably HMF and furfural). Therefore, these impurities can impair microbial growth, and severely decrease the yield, productivity and stability of the bioconversion process [1]. In addition, impurities can also have negative effects beyond the microbiological process: a complex syrup is indeed more likely to contain catalyst poisons, which, if not eliminated at the fermentation/purification step, can negatively impact downstream chemical transformations.

Second, in contrast with most 1G feedstocks, which contain a single sugar species (glucose or sucrose), LC sugars are by essence heterogeneous, and consist in a mixture of glucose, xylose, and various other hexoses and pentoses. Wheat straw consists of up to 37% of Cellulose (mostly Glucose) and 25% of Hemicellulose (mostly Xylose), and the hydrolysate will accordingly contain a majority of glucose and xylose, in a 2:1 proportion. This heterogeneity is difficult to manage in an industrial process, because classical industrial microbes do not process different sugars simultaneously and at the same rate, and have a strong preference for glucose [2]. Thus, making the best use of all sugars, while keeping the highest productivity over the whole process is one of the major goals of the project.

GBE has developed microbes performing the biochemical transformation of first generation sugars, such as glucose or sucrose, to IBN. The global purpose is to adapt these strains to WSH substrate. A first essential step is to make an assessment of the performances of current strains on this substrate, in order to have a detailed characterization of the starting point of the project. In the same time, the first experiments of directed evolution, aiming at the improvement of consumption of this substrate were initiated

2. IMPACT OF CLARIANT SUBSTRATE ON INITIAL STRAINS

2.1. Impact on growth phase

Growth was monitored in a series of strains, including IBN producing and IBN non producing strains. Growth was compared in GBE's standard process in a fed batch mode either with WSH, either with glucose or with synthetic sugar mixtures mimicking WSH composition. Growth inhibition could be detected, although the strength of inhibition was largely depending on the experimental conditions: whereas growth was fully inhibited when WSH was present in the initial batch in minimal

medium, inhibition was partial on a rich medium, and marginal or not observed when WSH was added in the feed at t=12TUs.

In contrast, whatever the experimental conditions, no growth inhibition was observed with a sugar mixture mimicking WSH sugar composition. Hence, growth inhibition is necessarily a consequence of impurities.

2.2. Impact on production phase

In the experiments described in part 2.1, a series of runs were conducted with IBN producing strains. These runs were conducted beyond the growth phase (around 40 TUs), and switched to production phase, by increasing sugar feed and changing other parameters such as pH. IBN production was followed by on line gas analysis with a Prima Pro mass spectrometer from Thermofisher. IBN production on WSH was in the same range as production on Glucose, as was observed previously. However, IBN overall production remained significantly lower on WSH, either because the initial production rate was nearly identical, but rapidly dropped over time (see Figure 1), or because productivity was lower from the beginning (but remained stable in this case).

In contrast, IBN production was identical or nearly identical with pure glucose and with a mixture mimicking WSH sugar composition. Hence, production inhibition is a consequence of impurities.

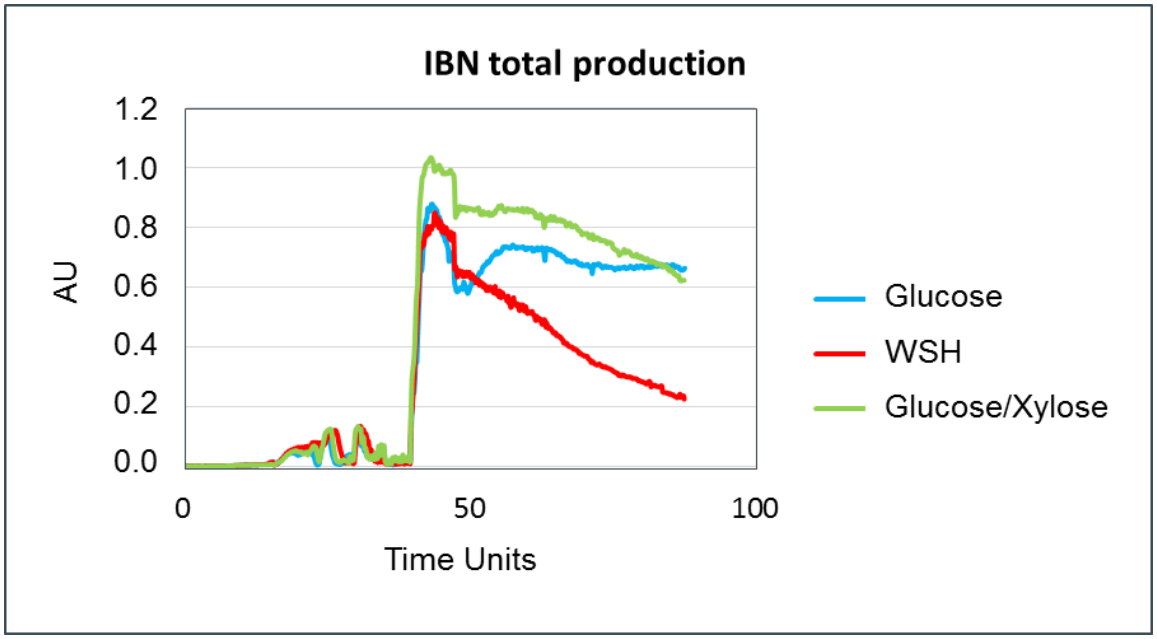

Figure 1. IBN production on Glucose, WSH and on a sugar mixture mimicking the sugar composition of WSH.

2.3. Sugar consumption rates

Glucose consumption rate appeared proportionated to growth and IBN production, with pure glucose, WSH and a mixture of Glucose and Xylose mimicking WSH sugar composition. Glucose uptake was nearly identical during the 50 first TUs, with the three substrates, and decreased with WSH around 50-60 TUs, when the IBN production decreased. Xylose consumption appears to be lower, but is observed as soon as xylose is introduced into the feed, with both WSH and glucose/xylose mixtures. Additional studies and measurement will be necessary to determine if glucose and xylose are consumed in proportion of their respective concentrations, or if a slight bias can be observed in favor of one of these two sugars.

3. ADAPTATION OF CHASSIS STRAIN TO CLARIANT SUBSTRATE

3.1. Adaptation of chassis

Early experiments showed that in certain experimental conditions, WSH could have a strong inhibitory impact on growth when added in the initial batch. These findings provided GBE with a method to select for resistant strains. *E. coli* strains were plated on medium containing a list of impurities identified in WSH. The concentration of these impurities was maintained high, in the range of 10-fold their concentration in the WSH. Colonies growing in these conditions were passaged several time on the same medium. After two months of selection, the first isolates were characterized in media supplemented with WSH, Glucose or synthetic sugar mixtures, in order to monitor their growth performances.

3.2. Performances of the selected strains

In order to maximize differences, growth and production experiments were conducted on minimal medium, with WSH added from t=0 TU (initial batch). As shown on Figure 2, a few weeks of growth in selective conditions were sufficient to restore a high level of growth on substrate. In order to test the new strains for their capacity to support a heterologous metabolic pathway with a high carbon flux, these strains were tested for acetone production. The acetone pathway, not present in *E. coli* in nature, is nevertheless much easier to handle than the isobutene pathway. Acetone production requires the overexpression of only three genes from *Clostridium*, which can easily be introduced into the new evolved chassis. Whereas the evolved strain seems to have lost some of its productivity on synthetic sugar mixtures, as compared to the original strain, it is now able to produce efficiently on WSH.

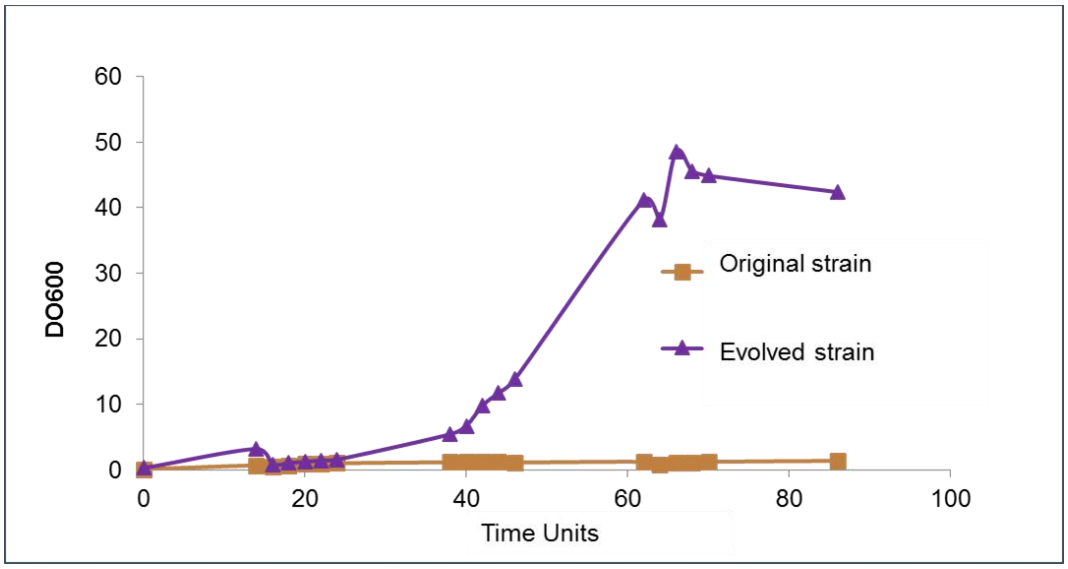

Figure 2. Growth on WSH before and after selection on medium with impurities. Experiments were conducted in conditions maximizing WSH inhibitory impact (see text)

4. Conclusion

A first series of experiment on WSH confirmed preliminary results obtained earlier. On short runs, and with the current chassis, growth and production performances are similar to the ones observed with pure glucose. However, detailed studies could confirm an inhibitory impact, which were maximized in certain experimental conditions, this inhibitory effect resulting in some case in the absence of growth. This phenomenon seems to be due essentially to impurities: mixtures of sugars mimicking the sugar composition of WSH did not impact growth and production, as compared to pure glucose. In addition, both glucose and xylose are consumed roughly in proportion of their concentration in the broth. Additional studies and measurement will be necessary to determine if a slight bias can be observed in favor of one of these two sugars.

The first directed evolution experiments aiming at adapting GBE's chassis to WSH were initiated, and in a few weeks, significant improvement were achieved, in conditions that maximize WSH' inhibitory effect. These experiments need however to be pushed forward, in order to match the results achieved with Glucose.

5. References

[1] Wang et al. (2013) Proc. Natl. Acad. USA 110: 4021-4026; Almeida et al. (2009) Appl. Microbiol. Biotechnol. 82:625–638.; Mills et al. (2009) Biotechnol. Biofuels 2:26.

[2] Luo Y. et al. (2014) Biotechnology advances 32:905-919 ; Kim S. M. et al. (2015), Metabolic engineering 30 : 141-148 ; Jung I.-Y. et al. (2015) Bioresource Technology 198:709-716 ; Vinuselvi P. and Lee S.K. (2012) Enzyme & Microbial Technology 50: 1-4; Sawisit A. et al. (2015) Bioresource Technology 19:433-441; Saha B. C. et al. (2015) Bioresource Technology 190:182-188; Jarmander J. et al. (2015) Microbial Crell Factories 14:51-63