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REWOFUEL

REsidual soft WOod conversion
to high characteristics drop-in bioFUELs

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Innovation Action Project

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Report on laboratory fermentation runs on RWH with matched strains enabling the MS3 performances

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Document Abstract

The REWOFUEL project aims to convert wood residues into high performances drop-in biofuels and value-added coproducts. The core of this technology is the biologically mediated conversion of residual wood hydrolysates (RWH) into isobutene (IBN), a platform molecule which is then converted into fuel components. Global Bioenergies (GBE) has been developing bacterial strains optimized for the production of IBN, and as part of this project is adapting the strains to produce IBN from RWH. Bacterial strains have been evolved and adapted to resist chemical impurities which were identified in RWH and are generated during the process of extracting the sugars from lignocellulosic materials. As a consequence, the strains are able to grow and produce IBN from RWH as a substrate, with a yield at 90% of the target set for REWOFUEL milestone MS3. Furthermore, continuous improvement of the IBN metabolic pathway has allowed reaching 55% of the target productivity on RWH.

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Table of Contents

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1 Introduction

Since its beginning in 2008, GBE has been developing microbial strains for the biochemical transformation of sugar compounds into isobutene (IBN). An entirely new metabolic pathway was designed and inserted into an optimized bacterial chassis by assembling genes adopted from various pathways and microorganisms. The modified bacterial cells are able to consume sugar molecules, such as glucose or sucrose, and convert them into gaseous IBN.

While the use of pure sugar substrates, referred to as 1st generation (1G) feedstocks, offers several advantages, such as high concentration of sugar molecules and absence of inhibitory compounds, there is a trend in the bioindustry aiming at complementing such substrates with resources derived from agricultural and forestry waste streams. The use of such 2nd generation (2G) feedstocks will improve feedstock availability as well as the overall environmental impact of the bioindustry. Thus, as part of REWOFUEL GBE is aiming to extend its technology for the utilization of sugars derived from forestry waste products, typically wood residues and leftovers such as wood chips, saw dust or tops and branches. In the REWOFUEL project, sugars derived from these products are referred to as Residual Wood Hydrolysates (RWH).

There are a number of challenges for producing IBN from 2G RWH substrates as opposed to 1G sugars, including the multiplicity and concentration of sugars present in the hydrolysate and the presence of potential inhibitor compounds which may be generated during the extraction process. As part of REWOFUEL, GBE aims to develop specifically adapted bacterial strains to cope with these challenges.

2 Results

2.1 Bacterial strains for the production of IBN

GBE's strains are derived from *Escherichia coli*, which is naturally highly efficient at metabolizing a variety of sugars. Sugars such as glucose are converted via glycolysis into the universal metabolite acetyl-CoA which then serves as a precursor for the synthetic, multi-step, IBN pathway (Figure 1). In GBE's *E. coli* strains the glycolysis and central carbon metabolic pathways have been modified in order to optimize the conversion of carbon atoms from glucose into acetyl-CoA with minimum release of CO_2, thus improving the overall yield of IBN production from sugar. The genes encoding the enzymes of the IBN pathway, a 6-step conversion of acetyl-CoA to IBN, are inserted into this chassis either on the chromosome or on multiple copy plasmids for increased expression.

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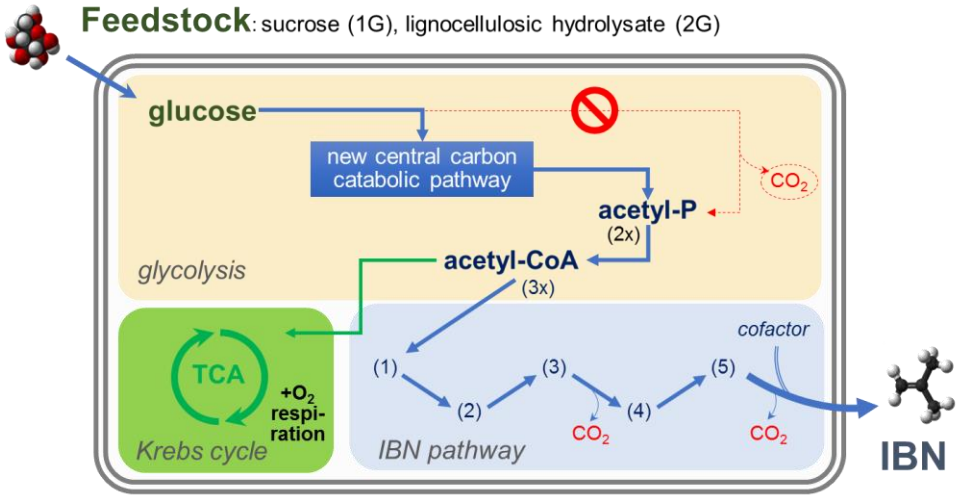

Figure 1. Metabolic pathway for the production of IBN from sugar substrates in GBE's modified *E. coli* cells.

2.2 Strategies for optimizing the production of IBN from lignocellulosic hydrolysates

At present, GBE's non-evolved bacterial chassis can consume >80% of sugars present in RWH. In a second stage of this project, the consumption of all sugar sources from RWH will be improved by using bacterial strains evolved to optimize their consumption of each sugar.

All 2G sugar feedstocks contain several impurities, including known inhibitors of fermentation, notably hydroxymethylfurfural (HMF) and furfural. Further mass spectrometry analysis of the hydrolysate performed at GBE's laboratory has revealed the presence of additional potential inhibitors at relatively high concentrations, including phenolics such as hydroquinone or coumaric acid. Surprisingly, HMF and furfural were found to have no significant impact on IBN production of GBE's strains. In contrast, hydroquinone and coumaric acid were shown to destabilize the production, and in the case of hydroquinone to result in a strong and irreversible inhibition of the key enzyme of the IBN pathway. Bacterial cells were evolved in long term chemostat experiments to be able to grow in the presence of increasing concentrations of HMF, furfural and hydroquinone. The obtained bacterial chassis was then equipped with the IBN pathway. However, IBN production with the new adapted strain grown on RWH was similar to that of a non-evolved strain allowed to grow on pure sugars (permissive conditions) and fed with RWH in production phase only after the bacterial culture had reached the required high cell density.

2.3 IBN production performances

Over the course of this project, specific targets have been set for IBN production performances, with final targets matching the requirements for commercial activities. These performances relate to (i) IBN productivity rates, (ii) yield of sugar to IBN conversion, and (iii) stability of the fermentation process. Because IBN is gaseous at process conditions and is continuously removed from the bioreactor rather than accumulated over time, extended production stability can be achieved compared to fermentation of non-gaseous products.

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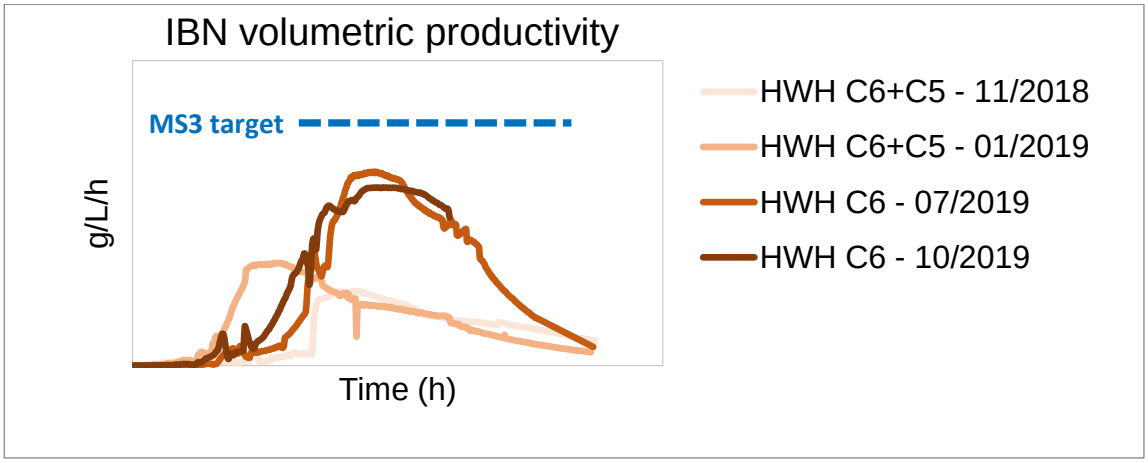
In REWOFUEL, 2 types of residual wood hydrolysates are being tested: (i) hydrolysates from softwood residues (RWH) provided by SEK and (ii) benchmarking hydrolysates from hardwood residues (HWH) provided by GIV. In November 2018, IBN production from RWH and HWH both showed similar performances compared to pure sugars, suggesting that impurities present in the hydrolysates had no significant impact on the IBN metabolic pathway

At the beginning of 2019, GBE has shown excellent progress on the IBN production capacity due to the development of a new evolved version of the key enzyme for IBN production (Ferulic acid decarboxylase; FDC) which was then fitted in the RWH adapted strain. This new FDC enzyme allowed for much higher production from hydrolysates of wood residues. Surprisingly, when testing the improved strains in July and October 2019 on wood hydrolysates, IBN production from HWH greatly increased but production from RWH showed only limited increase (Figure 2). IBN production at lab scale (1 L bioreactors) on HWH increased by 2.9-fold in productivity compared to November 2018, and reached a yield of 0.16 g/g of IBN produced per sugar (glucose, xylose) consumed (Table 1). These performances were equivalent to 54% of the target productivity and 80% of the target yield (0.2 g/g over 48 h) as set in MS3. In October 2019, IBN production from RWH at lab scale increased by 2.0-fold in productivity over 24 h with a yield of 0.12 g/g (Figure 2). Thus, performances on RWH had not increased as much as performances on HWH. This limited improvement is likely due to the presence of chemical impurities in the batches of RWH that were tested that prevented higher IBN production. However, in January 2020, a new highly concentrated batch of RWH was received and, when tested at lab scale, showed a further 1.8-fold increase compared to previously best performances, with a yield of 0.18 g/g (Figure 2, Table 1). Thus, at present performances on RWH are equivalent to 55% of MS3 target productivity and 90% of target yield.



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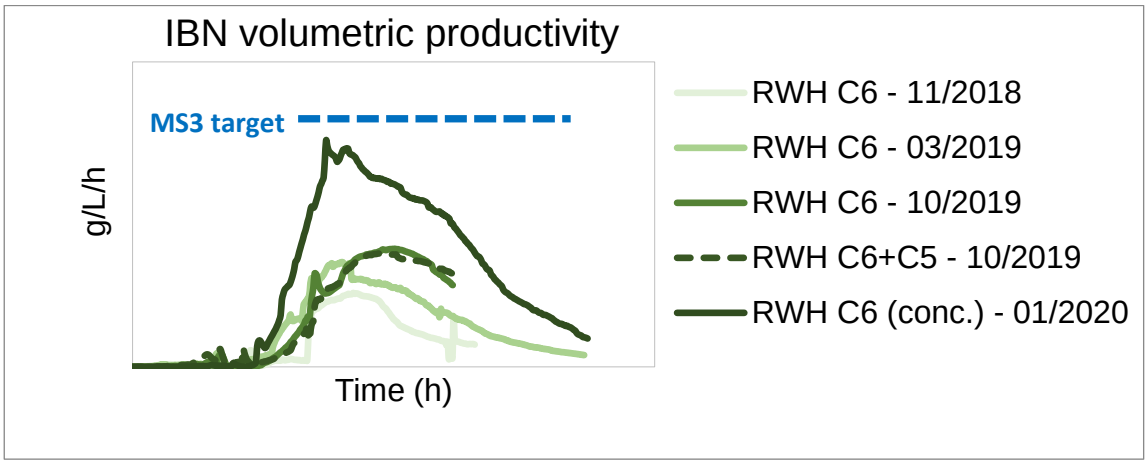

Figure 2. Increases in IBN production performances with various substrates between end of 2018 and 2019. RWH, residual softwood hydrolysate provided by SEK; HWH, residual hardwood hydrolysate provided by GIV for benchmarking activities.

Type of substrate	Duration (h)	Yield (g_{IBN}/g_{sugar})	%age of MS3 target
glucose base	24 48	0.21 0.23	 115%
hardwood hydrolysate HWH	24 48	0.18 0.16	 80%
residual softwood hydrolysate RWH	24 48	0.18 0.18	 90%

Table 1. IBN production yields

3 Conclusions and next steps

IBN production performances of GBE's strains have progressed significatnly in 2019. However, as of today, the performances obtained on wood hydrolysate are not yet as good as those obtained with pure sucrose or glucose. Thus, at this stage the expected performances of MS3 could not be reached within the timeframe initially set in the proposal, with current performances being at 55% of the target productivity and 90% of the target yield. These results are mainly due to the effect of inhibitor compounds present in RWH on IBN production. Indeed, impurities in hydrolysates appear to destabilize production over time, while interestingly the peak productivity is closer to MS3 target.

Currently, bacterial strains and fermentation protocols are continuously adapted to improve IBN production stability on RWH, and a new series of enzymes is being developed to overcome performance limitations. The latest evolved enzymes showed excellent potential which are likely to increase IBN strain performances on RWH within 2020. As part of REWOFUEL task T3.1, GBE will continue testing new strains with GBE's latest IBN producing enzymes, as well as test new more optimized/concentrated formulations of RWH.

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