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Introduction

The purpose of this project has been to demonstrate the viability and commercial readiness of an integrated biorefinery for low cost production of 1,4-butanediol (BDO), from biomass derived sugars—deliver the engineered strain and optimized fermentation process to enable the conversion of cellulosic sugars into BDO. Currently, BDO is derived from natural gas or petroleum via a number of chemical processes or from dextrose first generation sugars, by the GENO BDO™ process from Genomatica. 1,4 BDO is an important intermediate chemical with a global demand of about 1.7 million tons that is used in the production of a number of polymers including polybutylene terephthalate (PBT), Spandex (Lycra, elastane), and polyurethanes. Therefore, production of BDO from renewable resources, specifically from biomass is both desirable from a societal standpoint as well as offering the potential to be free of fossil fuel price volatility, availability, and environmental impact.

To this end, Genomatica has developed the GENO BDO process to produce BDO from sugar by harnessing microbial metabolism. Using constraint-based metabolic modeling, bioinformatics gene/enzyme identification and prioritization, low, medium and high throughput screening of genes and enzymes, metabolic engineering, fermentation process development, and BDO recovery process development, Genomatica has brought a glucose to BDO process to commercial scale and reality. This involved determining, via computational metabolic modeling, how to direct metabolic flux of carbon, redox, and energy towards production of a product not thought to be produced in nature. Then it was necessary to identify candidate genes encoding enzymatic activities sufficiently promiscuous to make BDO when given the appropriate substrates. A very large effort at optimizing this suite of *Escherichia coli* strains was required as well as building an optimized fermentation process. Commercial scale would not be possible

unless the BDO produced could be recovered and purified to the specifications required of fossil fuel-derived BDO. All of these were achieved in ongoing research and development with the goal being a cost-advantaged process to make this important chemical from renewable resources.

That project presented numerous challenges. For example, key enzymes identified, cloned, and expressed in BDO-producing *E. coli* are still capable of making native products if the suitable substrates are available. Optimized pathways still require sufficient flux to the final product, BDO, to prevent accumulation of byproducts. Figure 1 illustrates the basic parameters including overall stoichiometry, the required enzymatic steps not found in w.t. *E. coli*, and how those steps can be included in cellular metabolism. Strains and modifications to this process are evaluated on several levels **1)** titer, production rate, and yield of BDO; **2)** accumulation of undesired byproducts, **3)** complete consumption of sugar fed, and other metrics such as culture conductivity that can affect BDO recovery economics. How some of these tasks were accomplished and how challenges that arose along the path towards commercialization were dealt with are described in our publications on the BDO project (Yim, H., et al. *Nat Chem Biol.* 2011 7(7):445-52. and Barton, N.R., et al *J Ind Microbiol Biotechnol.* 2015 42(3):349-60.).

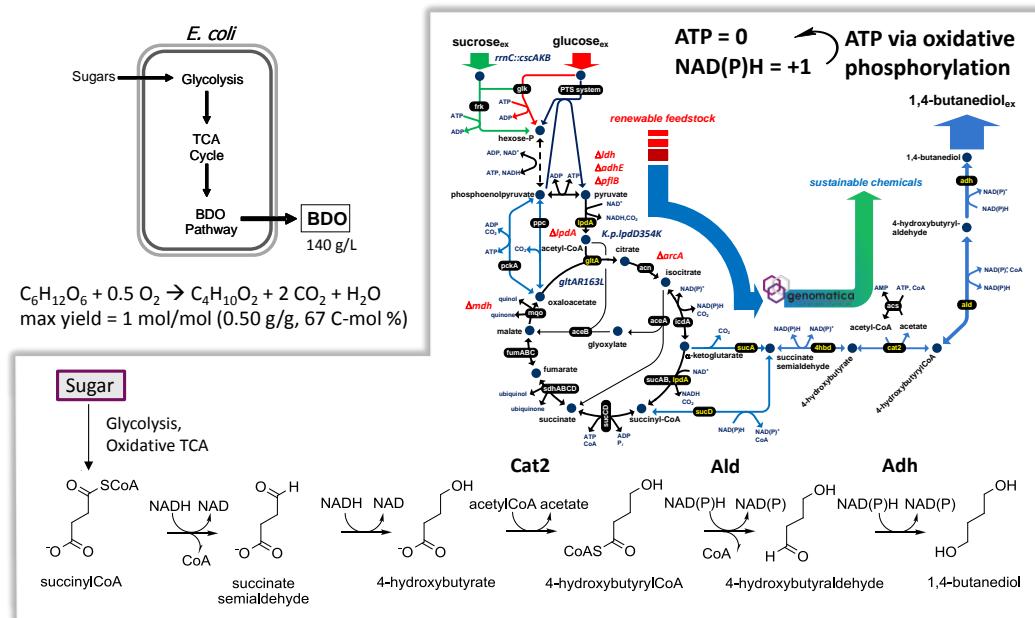


Figure 1. Producing BDO through microbial metabolism. Upper left, basic stoichiometry of bio-BDO, the 0.50 g/g theoretical yield is based on the pathways shown in the rest of the figure and this does omit the needs for biomass production in growing cells and energy conservation. Bottom, the published 1,4-BDO pathway from succinyl-CoA. Middle and right, the BDO pathway incorporated into *E. coli* cellular metabolism. Other pathways to produce BDO have also been identified and tested.

From 2011 to early 2015 Genomatica worked on achieving the goals and aims of the grant from the DOE for Biochemical Conversion technology to develop the capability of an integrated biorefinery. The concept is that lignocellulosic feedstocks can produce multiple streams derived

from cellulose, hemicellulose, and lignin. Development of a process to produce an added-value chemical such as BDO from lignocellulosic sugars can help support operation of a fermentation plant that might also use some of those feedstocks to produce a biofuel such as ethanol. This would be a bootstrap approach towards economical production of biofuels from biomass. For chemical production, research and development of biomass-derived sugars to produce a chemical such as BDO would offer **1)** increased feedstock flexibility could cushion market fluctuations in sugar prices and/or **2)** increase the available geographic space in which a BDO fermentation process can be deployed. That is, the latter can be strongly affected in regions with little or no agriculturally produced glucose.

Below are the specific aims proposed in the original grant; these constitute some of the basic necessities to develop scalable and commercial use of biomass feedstocks in the GENO BDO process. For example, *E. coli* normally metabolizes a variety of C6 and C5 sugars including glucose, xylose, and arabinose. However, *E. coli* does not normally metabolize all of these concurrently and instead shows a trait called diauxie in which some sugars such as glucose and fructose are used in preference to the other sugars such as xylose. Therefore, it was necessary to modify *E. coli* cells to simultaneously consume all the major sugars found in biomass hydrolysates. Each metabolically engineered strain is likely to have its own peculiarities in metabolism and uptake of sugar. Also, lignocellulosic biomass hydrolysates contain products derived from lignin degradation, from chemical reactions affecting sugars, such as xylose, and from other contaminants. All of these other chemicals can affect growth and metabolism, therefore, requiring strains or processes that minimize these effects. Finally, because lignocellulosic hydrolysates differ in chemical composition, differ in sugar concentrations and composition, and are complex and variable mixes fermentation processes also need to be optimized for these.

1: Improving the microbial conversion of cellulosic sugars to BDO.

To deliver commercially acceptable performance and enable scalable integrated biorefineries.

2: Characterizing and improving tolerance to cellulosic hydrolysate.

To deliver commercially acceptable performance and enable scalable integrated biorefineries.

3: Developing and optimizing a scalable fermentation process. Demonstrate the feasibility and scalability of integrated biorefineries.

Deliver strains and process for BDO from cellulosic sugars at titer ≥ 70 g/L, and productivity ≥ 2.0 g/L/hr at 30 L scale.

There is a lot of interest in developing lignocellulosic biomass as a renewable feedstock for both chemicals such as BDO and for fuels, starting with ethanol (EtOH). Considerable historical progress in pretreating biomass to make sugars available through enzymatic hydrolysis, optimizing EtOH producing organisms to grow on biomass and to co-utilize biomass sugars has been made. EtOH has a distinct advantage, recovery is through distillation that separates the product, EtOH, from the cells and precursors. Often this is through a simultaneous saccharification and fermentation process (SSF) in which lignocellulosic hydrolysates containing celluloses and hemicelluloses are simultaneously inoculated with cellulolytic enzymes and an EtOH fermenting organism. The product is distilled off (Figure 2). Conversely, bio-BDO synthesis

requires higher sugar concentrations that usually also means that saccharification of polymeric sugars must take place prior to fermentation (Figure 2, lower).

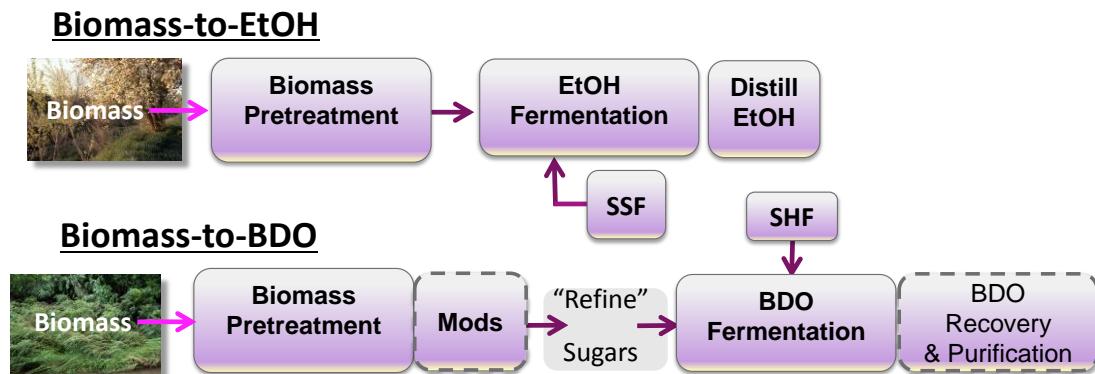


Figure 2. Schematic comparison of the basic processes for industrial (fuel) EtOH from microbes (upper) and for bio-BDO (lower). In contrast to EtOH, BDO requires biomass modifications (“Mods” that include saccharification (separate saccharification and hydrolysis, SHF) and increased monomeric sugar concentrations), and, finally, BDO recovery is very different from that of EtOH and requires the removal of impurities, water, and color from the BDO.

To pursue these goals and develop biomass as a feedstock in BDO production, Genomatica has harnessed its full range of technologies from computational modeling, diagnoses with various ‘omics methods, metabolic engineering, enzyme engineering, microbiology such as adaptive laboratory evolution, and fermentation process. A schematic of how these can be applied is depicted in Figure 3, below. Key to understanding this is the iterative nature of all of these. We do not perform metabolic modeling in isolation of metabolic engineering and fermentation process development. Instead, each of these provides constructive feedback to the others.

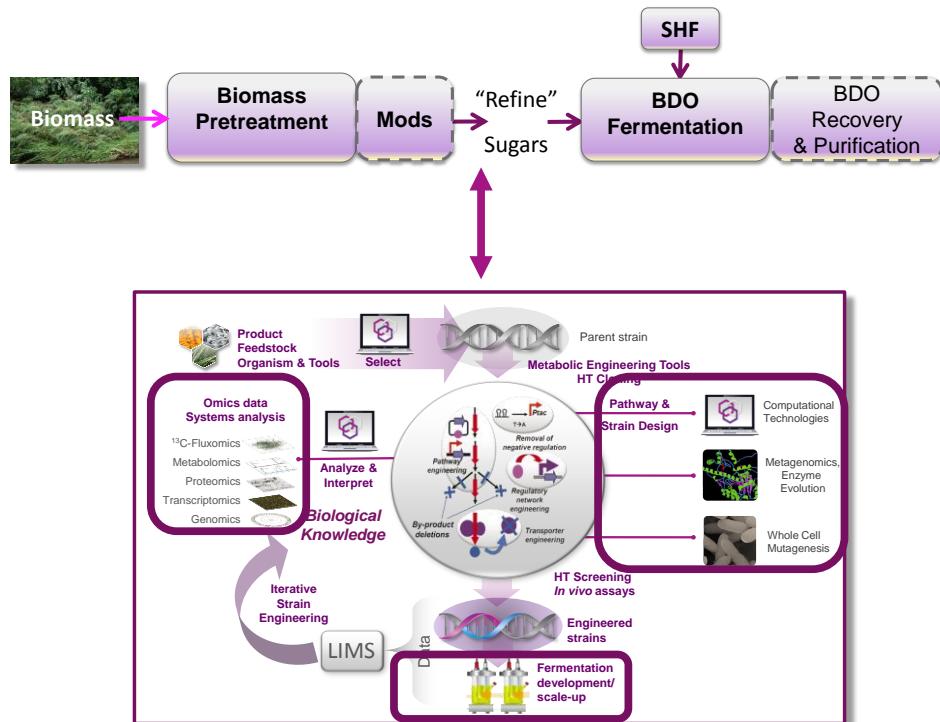


Figure 3. Schematic illustration of feedback between development of a biomass-to-BDO process and the core technological capabilities of Genomatica. Boxes highlight the main technological areas contributing to progress in this grant work.

Approach towards the specific aims and goals

Progress towards the aims of the grant was set in the metrics of titer, rate, and yield of BDO (T-R-Y). That is, achievable BDO titer in g/L of the fermentation, the rate in g/L/hr at which the titer was reached, and the actual process yield in g/g (BDO/fermentable sugars). The goals were set at T = 70 g/L and R = 2.0 g/L/hr at a 30 L scale. A proprietary yield metric was also included.

Reaching these goals required **1) strain engineering and improvement to make a BDO producing strain grow on mixed 5 carbon and 6 carbon sugars found in biomass hydrolysates, 2) strain engineering to optimize growth and yield on these feedstocks via directing flux towards BDO and also towards more efficient production and utilization of energy for the cell, and 3) fermentation process improvements to optimize the improved strains on biomass sugars.** Additionally, technical-economic analyses (TEA) were performed to understand the implications of each change and to formulate, with both strain engineering, fermentation, and BDO recovery the economic needs for use of biomass feedstocks with GENO BDO. The TEA work fed back into the others by demonstrating that impurities present in some biomass hydrolysates impact BDO recovery economics enough to require modifications in lignocellulosic hydrolysates independently of the effects of strain improvement. Therefore, a major result of this effort was the formulation of a minimum specification of biomass hydrolysates for biomass-to-BDO. Along the way, genes were identified and cloned after metabolic modeling predicted needs for energy optimization, strains were evolved for C6 – C5 sugar co-utilization with contributing genes identified via whole-genome DNA sequencing, and plans were formulated and tested for BDO

recovery improvements that would impact the economics of biomass feedstocks in the GENO BDO process. During the course of this work, we obtained biomass hydrolysates from 9 different suppliers representing a wide range of pretreatment technologies, feedstocks, sugar concentrations, and levels of purity.

Results

Sugar co-utilization

As described above, overcoming the diauxic response of *E. coli* BDO producing strains to various sugars found in lignocellulosic biomass derived hydrolysates was a critical goal to enable complete sugar utilization and to maximize process yield. An early example of a Genomatica-engineered BDO producing strain was compared with the w.t. parent, *E. coli* K-12 MG1655 for growth on an artificial 50/50 mix of glucose and xylose. As found by Monod back in the 1940s, marked diauxie was observed (orange curve in Fig 4 vs. inset from Monod, 1942); this means that MG1655 preferentially uses glucose in the medium and, once that is depleted, switches its metabolism over to xylose. However, the BDO producing strain (magenta curve in Fig 4) also displayed diauxie but much reduced to the parent. The small blip in the line for the BDO producer means that only a minimal change in metabolism was needed compared to MG1655 which ceased growth for at least 15 – 30 minutes to change expression of its genes involved in sugar uptake and metabolism.

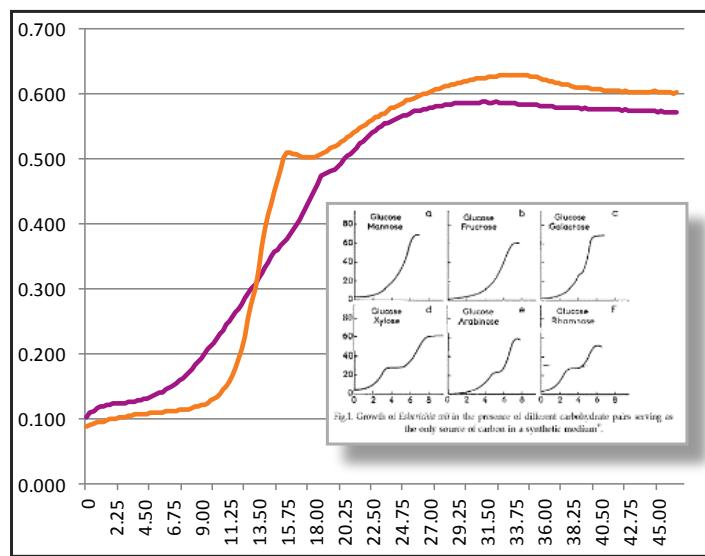


Figure 4. BDO-producing strains have a diauxic phenotype distinct from their wild type (wt) parent. *E. coli* MG1655, wt (orange) and *E. coli* MG1655-BDO producer (magenta) grown on a 50:50 mixture of glucose and xylose; x-axis is time (hrs) and the y-axis is OD_{600nm}. The inset is copied from Jacques Monod (1942) and represents the discovery of diauxie in *E. coli*.

At first, we did not know what mutation, unintended or a consequence of an intended change, contributed towards this phenotypic shift. However, we decided to exploit it based on the assumption that we were now 1 mutation closer to the desired phenotype of co-utilization. To

this end, we selected the BDO production strain in a proprietary chemostat design for growth in limiting xylose + extremely low levels of glucose. The glucose was added to avoid selection against glucose utilization as sometimes happens. This strategy was successful, within 400 generations the entire population in the chemostat grew faster and to higher optical density than the ancestral population. Clones were isolated from the evolved population and tested for alterations in diauxic sugar utilization. Some clones retained parental diauxie, we believe that they, however, became more efficient at taking up glucose and thus able to compete in growth rate with the rest of the population under selection. A minority of the strains did show a complete absence of diauxie. One of these clones was subjected to whole genome re-sequencing on an Illumina platform. That strain was found to carry at least 4 mutations that had been fixed. One mutation was in a global regulator believed to facilitate faster cell growth, a couple others didn't make any sense and one mutation was in a gene that could have been important. While this work seemed to confirm that, we tested by introducing the mutation into the same BDO production strain background. This change appeared to work as predicted by conferring a xylose-co-utilizing phenotype on an unmutated, naïve strain; we designated this genotype as "xylose utilizing mutant 1" or XUM-1. Strains engineered with XUM-1, including MG1655, all become glucose-xylose co-utilizers, indicating that XUM-1 is sufficient for this phenotype. During this process, the full genome sequence of the ancestral strain also provided a very likely candidate gene that could be responsible for the reduced diauxie in the BDO production strain lineage. As this genetic change is not essential for the XUM phenotype, one possible hypothesis is that it may have potentiated selection for XUM.

In the presence of the XUM-1 mutation, glucose and xylose were co-utilized (data not shown) while arabinose would still accumulate in the medium. An arabinose utilizing/uptake gene was also cloned into the biomass-to-BDO strain via integration into the genome. The resulting strain was capable of co-utilizing all three major biomass sugars, glucose, xylose, and arabinose. Figure 5 depicts one experiment demonstrating this phenotype. This was performed as a fed-batch fermentation in which the sugars are fed continuously throughout the fermentation experiment, 36 hours in duration in this example. If any of these 3 sugars was not utilized, then that sugar would tend to accumulate during this kind of fermentation. In that case, an increase in the slope of the line of one, but not all, sugars would be observed. Due to feeding programs and the inherent variability, the residual sugar lines in these fed batch fermentations do have some minor fluctuations but overall trends are clear. Therefore, not only did none of the sugars accumulate but all 3 maintained approximately equal levels throughout. This was the desired phenotype. Though this particular experiment was performed using a refined sugar mix, we have repeated this with biomass hydrolysates of several different glucose : xylose : arabinose ratios and co-utilization was stable and reproducible in all cases.

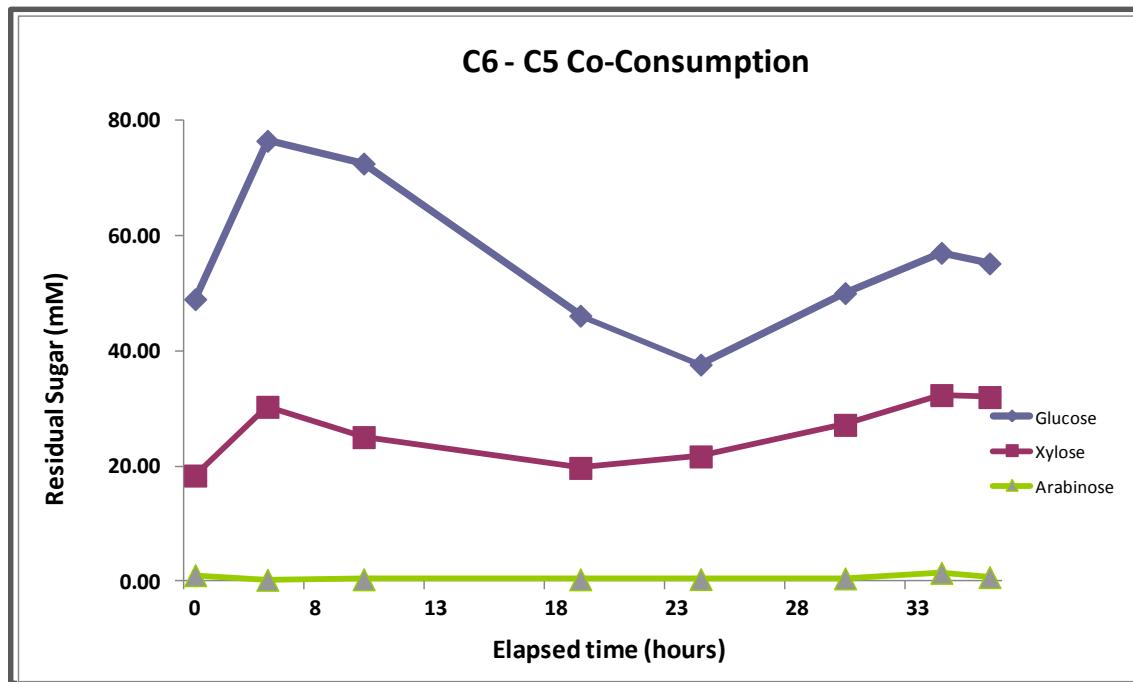


Figure 5. BDO production strain with XUM-1 + arabinose uptake/utilization co-consumes all three major sugars. The strain had the XUM-1 genotype introduced along with the separate arabinose utilization gene. A fed batch fermentation at the 2 L scale was performed with a continuous feed of refined sugars at a ratio of approximately 3 : 2 : 0.01 (glucose : xylose : arabinose). Subsequent experiments with biomass hydrolysates have all confirmed this result (not shown).

These results demonstrated the wide applicability of our XUM-1 gene and the arabinose utilization gene as well. However, growth of the cells and yield of BDO on biomass hydrolysates still showed some room for improvement. To understand this issue, ¹³C flux analysis was undertaken. This diagnostic analysis works by using the very rare carbon isotope, ¹³C as a tracer. This work showed that diversion of metabolism to utilize xylose tended to limit energy availability to the host cell (data not shown). Therefore, further modifications of the strain were undertaken to reduce energy requirements for xylose uptake and metabolism by removal of genes encoding an energetically costly means of xylose transport and to modify, via enzyme engineering, a transporter for xylose that is more energetically efficient.

The features related to xylose utilization, uptake, and energy efficiency were all combined together on a genetic cassette termed “Biomass Utilization Genes” (BUGs) that allows for a 1 step process to convert any Genomatica BDO producing strain to an efficient glucose-xylose co-utilizer. This development, though late in the project, has allowed for a far greater rate of strain construction. Both of these developments, the XUM phenotype and incorporation of that into the BUGs cassette are two of the major achievements of this DOE-funded project because they enable a diverse range of strain options for biomass sugar feedstocks for 1,4-BDO and other chemicals that might be produced via *E. coli* fermentation.

Fermentation and hydrolysate composition

Originally, the DOE grant to Genomatica included one company, B1, as a partner. Prior to awarding of the grant, Genomatica reached an understanding with another provider of lignocellulosic hydrolysates, B2. During initial validation, both hydrolysate sources were used in fermentation runs for a visiting team of DOE and NREL officials. The material from B1, however, may have been overlimed and when added to bacterial culture medium components containing phosphate, a crystalline precipitate was formed. This and company priorities pointed towards working with B2 for the next couple of years. This was very fruitful as both groups explored ways to modify hydrolysates from the B2 process, which uses agricultural residue as a feedstock, to optimize performance. From the start, some changes were required due to the nature of BDO fermentations. Fermentable sugar concentrations need to be >600 g/L due to a very large impact on fermentation scale and BDO recovery economics.

Figure 6 summarizes two years of work in Genomatica strain engineering along with fermentation process development combined with the experience built from >14 lots of lignocellulosic hydrolysate provided by B2. Most obvious is a >6 fold improvement in BDO titer during this time from 14 g/L to ~90 g/L. Results from only two strains are depicted. The validation strain, H, is an early gen 1 BDO strain with no modifications for sugar uptake and utilization while the later strain, Super G, has had the XUM phenotype added as well as improvements in arabinose utilization. Super G, however, did not have the full BUGs cassette, with its improvements in energy conservation on C5 sugars and also lacked some other improvements in BDO strains that were under evaluation at that time. While the original benchmark value in Fig 6A (dark filled in circle) used an early lot of B2 lignocellulosic hydrolysate, the later fermentations all used a B2 lot that had higher sugar concentrations (>600 g/L) and improved impurity reduction. Therefore, the difference using lot 014/12 between early strain H and later strain Super G highlights major improvements in strain performance and also demonstrated that a strain built around optimal BDO production and sugar co-utilization could, in fact, approach commercially practical metrics. In fact, BDO titer at this point reliably exceeded the original grant goal of 70 g/L and productivity was approaching the goal of 2 g/L/hr. Yield, in contrast, lagged behind due, in part, to the less optimal sugar uptake energetics combined with several other areas in the BDO pathway that were open to optimization in expression and enzyme engineering.

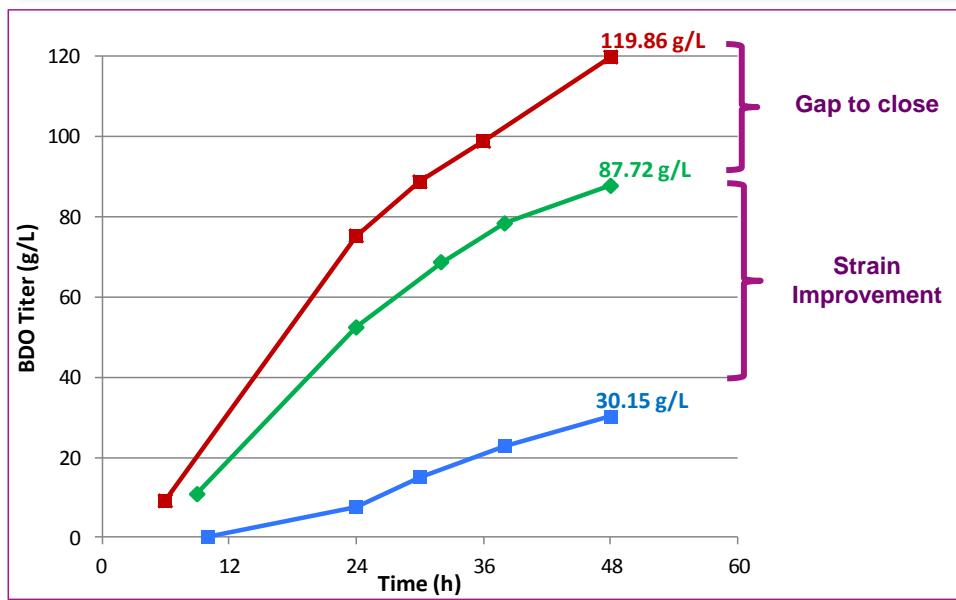
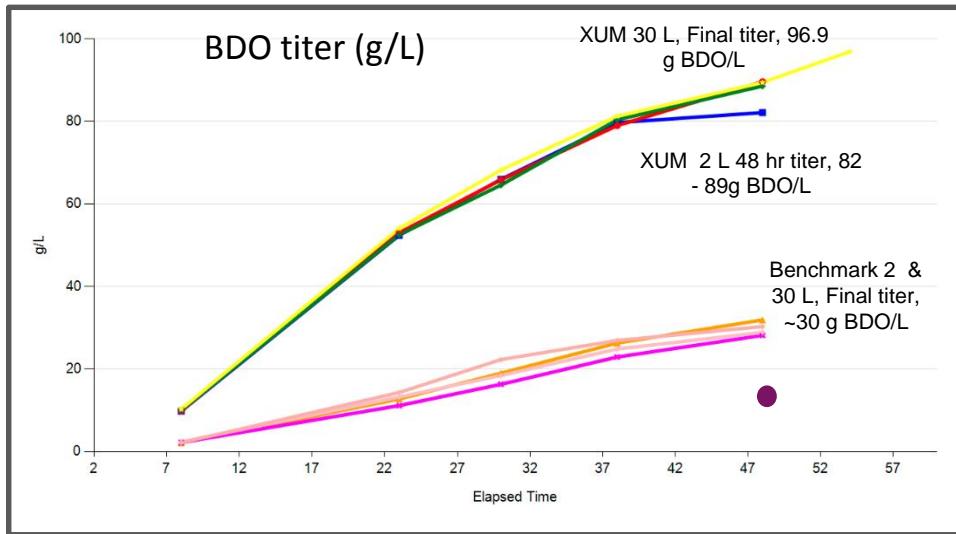


Figure 6. Comparison of BDO titer improvements due to strain engineering and hydrolysate composition. **A.** The large dark magenta filled circle is the 48 hr BDO titer in early pre-lot 001 B2 hydrolysate (~180 g monomeric sugar/L) with the original benchmark strain, "H". The other curves are BDO titer values over 48 hrs in both 2 L and 30 L fermenters. All used B2 lot 014/12 and two different strains, "H" (lower curves) and "Super G" (upper curves). **B.** Some of the same data from 6A for H (blue curve) vs. Super G on B2 lot 014/12 lignocellulosic hydrolysate (green curve) combined with Super G on C hydrolysate (hardwood feedstock treated with HCl) (red curve).

This performance (Fig 6A) was limited in two possible ways, first, feed sugar concentrations were still too low at 600 g/L to maximize BDO titer in a fed batch fermentation and second, though several critical impurities had been significantly lowered in B2 lot 014/12, impurity load still combined with sugar concentration could limit BDO performance—especially rate of BDO

production and final process yield. The actual potential of the intermediate strain, Super G, was tested with a very concentrated and clean hydrolysate feed from the supplier C. This material was derived from hardwood using an acid-based pretreatment. Total monomeric sugar concentration was considerably in excess of 700 g/L and was predominantly glucose, mannose, galactose, and xylose. Impurities were nearly absent and color was almost as clear as refined sugar. Not surprisingly, performance increased considerably (Fig 6B, see the red curve for C). At a 48 hr titer of 119 g/L and total productivity of nearly 2.5 g/L/hr, these key metrics were exceeded. Performance on pure glucose for this strain was similar or slightly behind (not shown). “Gap to close” on Fig. 6B is, therefore, an estimate of the latent capability of that strain genotype for BDO production. At this point strain engineering that resulted in the BUGs cassette and several other genetic changes to effect optimized expression of BDO pathway genes and minimize carbon loss to byproducts were engineered into a new lineage, “CI”.

At this juncture, two pursuits were deemed important. **1)** The DOE Program Officer suggested that Genomatica test a wider range of lignocellulosic hydrolysates representing other pretreatment technologies. This was highly desirable because it would aid in coming up with a key requirement for a large-scale, standardized process. **2)** A specification for lignocellulosic biomass hydrolysate composition that would be compatible with the unique requirements of a biomass-to-BDO process.

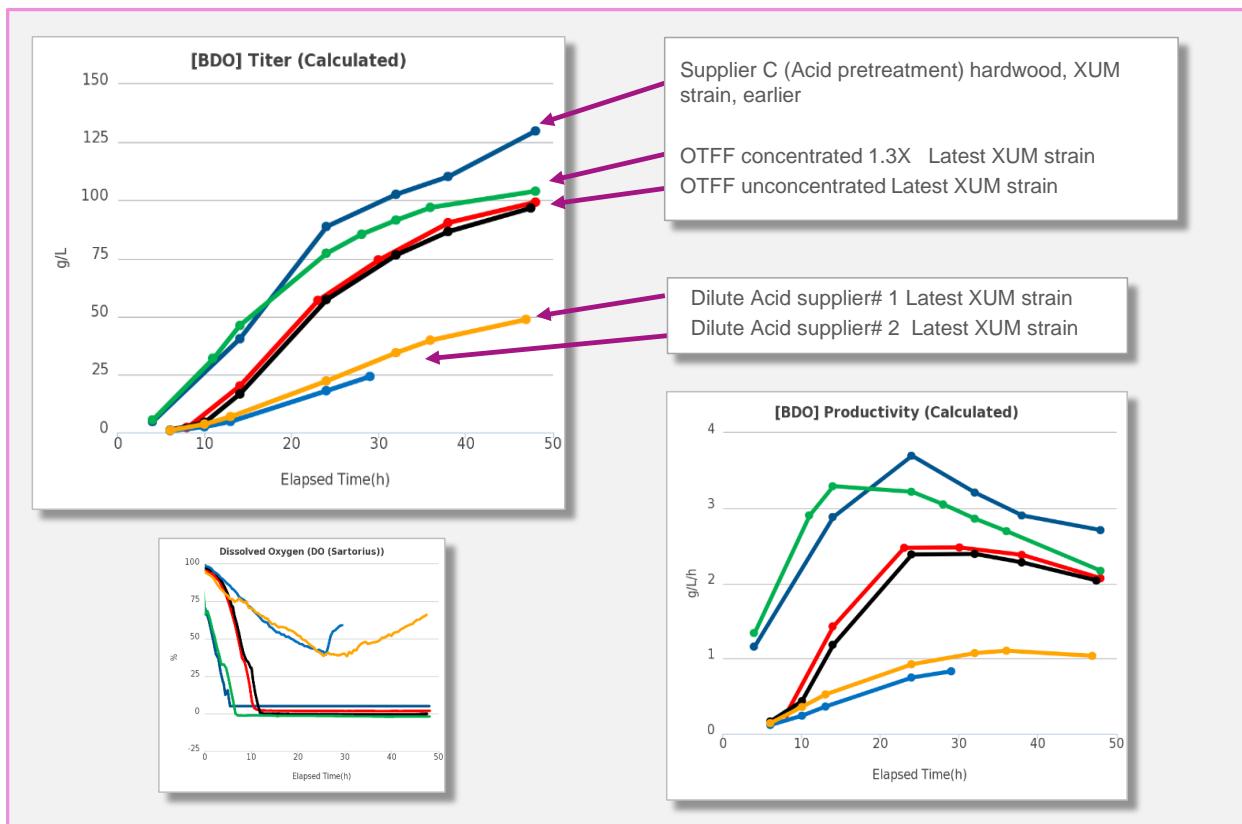


Figure 7. Comparison of several hydrolysate sources on BDO production. Hydrolysates were obtained from several suppliers: C, acid pretreatment already described above as a positive control; OTTF (Old Town Fuel & Fibre, not currently operating) a pulp-paper process from wood and agricultural residue; Dilute Acid supplier #1; and Dilute Acid supplier #2, both from either wood or agricultural residue. Additionally, OTFF was shipped at very close to 600 g sugar/L, some was concentrated at Genomatica on a Roto-Vap; concentration of 1.3X was achieved to raise the sugar concentration to ~750 g/L. BDO titer (g/L), Productivity (g/L/hr), and dissolved oxygen during the fed batch fermentation (% saturation) are depicted.

These tests were done and some of the data are summarized in Figure 7. Data for hydrolysate from supplier C (also see above) are included in this comparison to indicate the range of maximum performance on high sugar concentrations. Unfortunately, exact sugar concentrations vary considerably and both dilute acid pretreatment suppliers provided biomass sugars at <200 g/L. One promising source was Old Town Fuel and Fiber (OTFF) that used a pulp and paper process having converted an older facility in northern Maine towards production of lignocellulosic feedstocks. The OTFF material arrived at roughly 600 g/L and was assayed at both this concentration and a small amount was concentrated at Genomatica to measure the effects of dissolved sugars on metabolism and BDO production. During the course of these fed-batch fermentations, numerous parameters were and are measured. Shown in Fig 7 are results of two of those: dissolved oxygen (as a % of saturation), this gives an accurate picture of respiration and cell stress. Also, conductivity of the fermentation broth is measured (data not shown) to understand key ion (salts, etc.) concentrations that accumulate in fermentations. These impurities or unwanted byproducts are measures of both the quality of the hydrolysate feedstock and of how well the BDO pathway was working.

To summarize the key points in Figure 7: sugar concentration in hydrolysates of low concentration is a driver of titer and rate but not byproducts nor cell health (compare OTFF with 1.3 X OTFF). Conductivity predicts cell health and overall performance (dilute acid suppliers #1 and 2 with OTFF or C). That is, failure to reach a dissolved oxygen of 0% (or close to this value) indicates that the cells in the fermentation are stressed and/or dying and not capable of increasing respiration. One of the dilute acid supplier lots (#2) shows a rise in dissolved oxygen with time; this can't happen in a growing and metabolizing culture and is a marker for cell death in the Genomatica process. Both dilute acid suppliers #1 and #2 fermentations were cut short because of a failure for the culture to grow or metabolize better.

Data like these and from other experiments allowed us to write a specification for biomass hydrolysates in a biomass-to-BDO process. The specification is necessary to insure economical BDO recovery after fermentation as well as to allow performance in fermentation sufficient to reach commercial targets. Key aspects include maximizing sugar concentrations and minimizing dissolved ion concentrations. Therefore, an all-encompassing concept of tolerance to hydrolysate, which may work in biomass-to-ethanol processes, misses the requirements of a biomass-to-BDO process.

Another supplier, “A”, of lignocellulosic biomass hydrolysates was identified with a pulp and paper technology. This supplier used a feedstock of agricultural residue with a modified, proprietary pulp and paper pretreatment process to provide sugars at very high concentrations and low levels of other impurities. Figure 8 is a representative 30 L fermentation using sugars from “A”. Furthermore, strain Cl, developed for several of the fermentations in Figure 7 was used for this experiment. Strain Cl has the BUGs cassette, optimized BDO pathway expression, improved energy conservation, and genetic modifications to maximize g/g yield of BDO. Clearly, the BDO titer and rate performance shown in Figure 8 are very high compared to original targets. Yield approached 90% of the conventional sugar to BDO strains. This fermentation was run to depletion of the sugars and the residual glucose and residual xylose plots show very close matches in the rate of depletion of the two principal sugars found in supplier “A” hydrolysate.

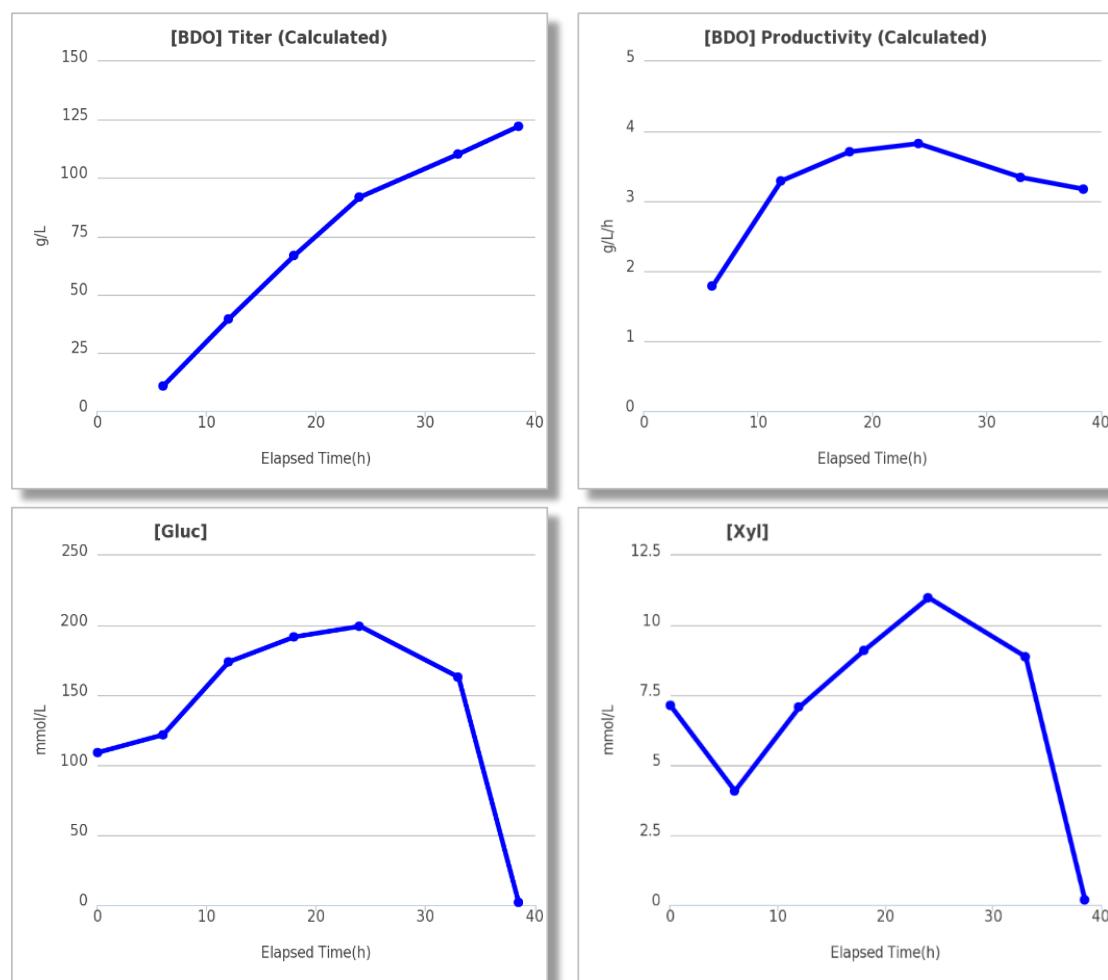


Figure 8. BDO production on supplier “A” biomass hydrolysate. Fed batch fermentation at 30 L scale was performed using biomass-to-BDO strain Cl (optimal pathway gene expression, improved aerobic metabolism and other changes relative to Super G or other strains). BDO titer (g/L), Productivity (g/L/hr), residual glucose (mM), and residual xylose (mM) are depicted.

While future biomass-to-BDO strains may incorporate even more changes than found in “C” the results with this strain on a variety of feedstocks, especially from supplier “A” go a long ways to demonstrate the commercial practicality of this process. Depending on economics, geography, and other considerations, this bio-BDO process could be applied to using renewable feedstocks with potentially fewer issues in geographic availability compared to corn sugar.

Response of BDO producing strains to hydrolysates

Several of the biomass hydrolysates tested with various BDO producing strains demonstrated varying levels of apparent toxicity. Some, such as preliminary samples from the dilute acid pretreatments and some of the lots provided by supplier “B2” early in the project directly inhibited cell growth and blocked or suppressed BDO production. Some efforts to adaptively evolve tolerance to the more toxic B2 hydrolysates were made but these efforts were ceased when it became apparent that even 100s of generations were not yielding improved tolerance. When this happens in these kinds of experiments, typically multiple genes must simultaneously mutate or the total concentration of the toxic material is too high (antibiotic development call this phenomenon “mutation prevention concentration”). It probably wasn’t the latter as diluting the material did not lead to improved results.

Therefore, different strategies were employed. Using ‘next-generation sequencing’ technology we assessed the transcriptional responses to different hydrolysates using high-coverage RNASeq. The two hydrolysates tested differed substantially in the types and numbers of genes that changed expression in the experiments. Performance of these in fermentation had also differed and this raised the suspicion that hydrolysate toxicity mechanisms may differ by 1) feedstock, 2) pretreatment technology, and 3) the sum of multiple factors. These results make a lot of sense, BDO strains tested are *E. coli* strains and these particular strains are not naturally exposed to high concentrations of lignocellulosic hydrolysates except as food coming into the gut—rarely are hemicellulose or lignin broken down into toxic components and the impurities introduced in pretreatment differ from those in our diet.

Finally, the sort of response observed in RNASeq immediately suggested other approaches to increasing tolerance to hydrolysates. One of these is trackable multiplex recombineering (TRMR). This method allows creation of many thousands of genetic modifications such as alterations in transcription of genes. TRMR is trackable because each modification is barcoded with a nucleotide sequence that can be identified by next generation sequencing. Selection or high-throughput screening allow testing very large numbers of modifications.

BDO Recovery

The chemistry of 1,4-BDO requires that water or other impurities from either fermentations or chemical catalysis be removed from the BDO. For the GENO BDO process, Genomatica engineers have devised an economical process to recover BDO of a purity equal to petrochemical BDO. This process however, can be affected by concentrations of dissolved solids, ionic and otherwise, in the final fermentation broth. Therefore, fermentations with some

hydrolysates lead to accumulation of other components of the fermentation broth that affect BDO recovery performance, and economics. Scaling up equipment can allow BDO recovery at purity meeting GENO BDO process (and also petro BDO) standards but at higher capital costs. Therefore, in the last phase of the grant work, several modifications to BDO recovery processes were identified and tested. This effort will enable inclusion of biomass-based feedstocks with the GENO BDO process for future licensees of Genomatica technology.

Summary

This project has demonstrated the level of commercial readiness for production of the industrial chemical, 1,4-butanediol (BDO), from lignocellulosic biomass by engineered *E. coli*. Targets were BDO titer, rate, and yield (TRY) and growth in lignocellulosic hydrolysates (Hz). A range of Hzs were used to assess limitations for biomass-to-BDO. Via adaptive evolution methods, whole-genome sequencing, and introduction of identified target genes, strains co-utilizing C5/ C6 sugars were made. The composition of Hz versus TRY led to a modified Hz composition. This was used in partnership with the DOE to redirect the project to focus on **1)** several biomass Hz from new suppliers, **2)** Hz specification due to the characteristics of the Genomatica BDO process, **3)** a gene cassette to engineer any BDO producing strain for biomass, and **4)** modified BDO recovery to more economically recover BDO at industry specifications. BDO TRY and growth of the *E. coli* strains were predictable based on Hz composition from several suppliers. This defined metrics for biomass Hz composition to achieve BDO TRY along with internal TEA to evaluate the economic potential of each modification to strain, Hz feed, and process. An improved biomass-to-BDO production strain reached BDO T-R in a 30 L fermentation above original objectives. Yield approached the proposed Y and modifications to BDO recovery were demonstrated. Genomatica is now in the position of being able to incorporate biomass feedstocks into the commercial GENO BDO process.