A novel fermentation strategy for removing the key inhibitor acetic acid and efficiently utilizing the mixed sugars from lignocellulosic hydrolysates

Final report for the COI grant with Synergy Parametrics

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This grant was requested by Synergy to accomplish two tasks. First, Synergy wanted us to develop a novel fermentation strategy to remove the key inhibitor acetic acid that is found in lignocellulosic hydrolysates and then convert the liberated mixed sugar mixture into ethanol. Second, Synergy wanted us to detail what future studies should be done for a grant to the Department of Energy to request additional grant funding for future studies. Both of these tasks were completed and are detailed in this report. The University of Georgia Research Foundation has filed a patent application on the technology that was developed.

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REPORT

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1. Introduction

Lignocellulosic biomass can potentially be used to diversify our current fossil-energy based systems for fuel, power and products (U.S. DOE). One promising use of lignocellulose for liquid fuel is in the microbial production of ethanol. There are many technical roadblocks that must be overcome, however, to generate a product such as ethanol from biomass. Lignocellulosic biomass must first be broken-down into its constituents, and a key technical challenge lies in the resulting complex mixture. This mixture contains sugars which individually but not collectively are suitable for fermentation and inhibitors that reduce the growth of fermenting microorganisms. Because the unit value of chemical products derived from biomass (e.g., ethanol) is generally low while the potential market is large, the economic viability of such processes depends on the *yield* and *productivity*. Achieving high yield demands that all biomass components be converted, while high productivity requires that the complex conversions be accomplished quickly. Several organisms have been proposed for the fermentation of lignocellulosic biomass into products such as ethanol, including *Escherichia coli*, Lactic acid bacteria, *Saccharomyces cerevisiae*, and *Zymomonas mobilis*.

Biomass hydrolysis leads ultimately to a mixture of hexoses (e.g., glucose, mannose, galactose) and pentoses (e.g., xylose, arabinose). Hydrolysis also leads to the formation of acetic acid which is a known inhibitor to any of the microorganisms that might be used to ferment the sugars into products such as ethanol. The fraction of pentose sugars which compose biomass is significant; for example, 12% pentose sugars have been reported for *Pinus* spp. and 26% for *Populus* spp. (Saddler and Mackie, 1990). In order to achieve high yields and productivities, both pentose and hexose fractions must be fully and efficiently utilized. While *S. cerevisiae* is presently the most widely used microorganism for ethanol production, industries have considered *E. coli* for lignocellulose-derived ethanol because of its metabolic flexibility and because current technology generates a hydrolysate with no greater than 10% sugar concentration that in turn would generate a low concentration of ethanol (<5%), which is within the tolerance of this species (Taherzadeh et al., 2001). However, current technology does not adequately convert sugar mixtures, and furthermore it does not overcome inhibition caused by the presence of acetic acid.

The efficient and *simultaneous* conversion of pentoses and hexoses is a significant hurdle to the economic utilization of biomass hydrolysates for the generation of *any* fermentation product. The central problem is that either the desired microorganism consumes the sugars sequentially (e.g., first glucose and then xylose) or the organism is unable to utilize specific sugars at all (e.g.,wild-type *S. cerevisiae* and xylose). Although the inability of microorganisms to utilize specific sugars such as xylose effectively is most commonly associated with fuel ethanol production, the formation of other fermentation products (butanol, succinic acid, lactic acid, pyruvic acid, etc.) from sugar mixtures would similarly be greatly enhanced by designing a process which uses both sugars effectively. Indeed, in a recent comprehensive review (Zaldivar et al., 2001), the authors succinctly conclude "the lack of a microorganism able to ferment efficiently all sugars released by hydrolysis from lignocellulosic materials has been one of the main factors preventing utilization of lignocellulose".

Essentially two strategies have been applied to ameliorate the problem of simultaneous pentose and hexose consumption, exemplified by xylose and glucose. One strategy has been to introduce genes involved in xylose consumption into an organism which does not natively have this ability but can generate a desirable product. For example, researchers have long studied the consumption of xylose by the common yeast *S. cerevisiae*, and the heterologous xylose

reductase, xylitol dehydrogenase and xylulokinase genes fused to glycolytic promoters have been successfully integrated into the yeast chromosome (Ho et al., 1998; Sedlak et al., 2003). A second strategy is to alter the cellular machinery which normally prevents xylose consumption in the presence of glucose. For example, a mutation in the *ptsG* gene of *E. coli* will reduce glucose-mediated repression of xylose consumption (Dien et al., 2002). Strategies to consume sugar mixtures have sought to develop a *single* organism that can "do it all".

Single organism strategies to convert xylose and glucose simultaneously suffer from considerable limitations. One limitation is that despite the introduction of the genetic apparatus to consume both sugars, glucose remains the preferred substrate. Thus, the consumption of the sugars is asynchronous, and xylose invariably remains when glucose has been consumed. In batch culture with the *E. coli* ethanologenic strain "K011" grown on a hemicellulose hydrolysate, only 11% of the xylose was consumed after 24 h, while 80% of the glucose was consumed (Barbosa et al., 1992). Though removal of the *ptsG* improves xylose consumption in the presence of glucose, 40% of the xylose remains when the glucose is depleted (Dien et al., 2002). Similarly, genetically engineered *S. cerevisiae* containing genes to consume xylose still consumed less than 25% of the xylose by the time glucose was depleted (Sedlak et al., 2003). Even adding xylose isomerase to convert xylose extracellularly did not solve the problem and most of the xylose remained after the glucose was completely consumed (Chandrakant and Bisaria, 2000). Approaches using "evolutionary engineering" have significantly improved the rate of xylose consumption (Kuyper et al., 2005), but have not prevented the diauxic behavior when using sugar mixtures (i.e., first glucose consumption, then xylose). Another organism of interest in ethanol production, *Z. mobilis*, also does not naturally consume pentoses. Introduction of genes for the xylose metabolism pathway similarly does not prevent this species from consuming xylose much more slowly than glucose (Zhang et al., 1995b). Because both sugars are not consumed effectively in any of these single-organism processes, the productivity of the process is suboptimal. This asynchronicity particularly reduces a single microorganism's ability to convert a real hydrolysate having a temporally varying concentration of each sugar. Faced with such a variable substrate stream, yet using a single organism which has a limited ability to adjust its ratio of glucose and xylose consumption rates, the process will invariably lead to one of the sugars not being effectively consumed. It is not currently possible for one organism to "adjust" its rate of consumption to two substrates in order to match fluctuating concentrations that would be encountered in a real process.

Another shortcoming is that a single strain constructed which contains both sugar consuming abilities tends to be unstable. An *E. coli* chemostat study demonstrated that the presence of both sugars caused over time an increase in the by-product (and inhibitor) acetic acid, which ultimately led to a 20% decrease in ethanol yield (Dumsday et al., 1999). A third significant disadvantage in current one-organism processes is that the metabolic pathways to convert glucose into a desired product at optimal yield and productivity do not generally correspond to the metabolic pathways to convert xylose into the same product. Ideally, a process converting xylose and glucose (and other carbohydrates) simultaneously into a single product would permit these pathways to operate *independently* of one another, with glucose metabolism not influencing xylose metabolism and vice versa.

As noted with real biomass the situation is unfortunately further complicated because hydrolysis invariably generates compounds which inhibit the subsequent conversion of sugars by fermentation. While inhibitors can be reduced by judicious design of the hydrolysis process or by improvements in the biomass itself, it does not appear feasible to eliminate all inhibitor generation. Since xylose is acetylated in lignocellulose (Timmel, 1967; Chesson et al., 1993),

acetic acid is an unavoidable product of hemicellulose depolymerization. Although acetic acid inhibition depends on the strain and process, a concentration of only 0.08% has been demonstrated to inhibit a subsequent fermentation to generate ethanol (van Zyl et al., 1991). Generally acetic acid reduces xylose conversion more than this inhibitor affects glucose conversion. In *S. cerevisiae* engineered to metabolize xylose, for example, acetic acid reduces ethanol yield from xylose by 50% (Helle et al., 2003). Acetic acid itself and not merely the pH causes the inhibition. Therefore, base neutralization traditionally applied to acid treated lignocellulosic hydrolysates does not eliminate the inhibitory affects of acetic acid. Not only must a process be able to handle varying mixtures of sugars as described above, but it must also be able to contend with the fluctuating presence of some inhibitors such as acetic acid without loss of yield or productivity. *The key factor in the success of a fermentation process to generate fuel ethanol (or any biochemical) from lignocellulosic biomass is the robustness of the fermentation organism(s) and process.*

We propose an entirely different, novel strategy for the efficient co-utilization of sugar mixtures which will allow them to be consumed simultaneously and *independently*. Our strategy permits each sugar to be converted into the desired product at each one's maximum yield, and the process adapts to fluctuating sugar concentrations without leading to the accumulation of any one sugar. Moreover, the approach can readily be extended to remove inhibitory compounds from hydrolysates.

The following *description* of the concept of substrate-selective uptake considers two of the important sugars found in lignocellulosic hydrolysates, the pentose xylose and the hexose glucose, as well as the primary inhibitor, acetic acid. The concept is not limited only to these two substrates nor this specific inhibitor, and as part of the proposed research and development plan, we will develop strains and processes to handle all the principal carbohydrates in lignocellulose: glucose, xylose, mannose, galactose and arabinose. We plan to develop strains of *E. coli* and *S. cerevisiae* although the strategy is also not limited to these microorganisms. Finally, we will apply the strategy specifically to the production of ethanol from hydrolysates using these two microbial species, although the strategy is not limited to this particular product. Therefore, the technology developed will be broadly applicable to all biomass, other substrates and other organisms. These characteristics exemplify the impact of this research.

The concept centers on the fact that we can readily "design" a single strain that will *only* utilize xylose, glucose or acetic acid. Such a strain has "substrate-selective uptake" since it is selective in what compound it is able to consume from a given mixture. For example, by deleting a key gene in the xylose uptake pathway, a strain of *E. coli* can be constructed which is *unable* to consume xylose. In a fermenter containing both xylose and glucose, such a "glucose-selective" strain would never consume xylose but would consume glucose normally. The glucose-selective strain should be completely *unaffected* by the presence of xylose. Similarly, a strain can be constructed which is unable to consume glucose. Placed in a fermenter with xylose and glucose, this "xylose-selective" strain should only consume xylose. The advantage occurs when the two strains are used *together* in one bioreactor. Inoculated simultaneously into a medium containing glucose and xylose, each strain will act on one sugar alone and be unaffected by the presence of the other sugar or the other strain. These benefits were clearly established in preliminary research.

2. Results/Technical Feasibility

As part of preliminary research efforts, we have completed several experiments which demonstrate "proof of concept." These experiments addressed the following three questions: 1) Can a synthetic mixed sugar solution of glucose and xylose be efficiently consumed using the multi-organism approach? 2) Can this approach be used to accumulate a model product? 3) Can this approach be applied to the removal of an inhibitor, acetate, selectively from mixtures of xylose and glucose?

To answer the question of whether this multi-organism approach can effectively consume synthetic mixed sugar solutions, we first tested substrate-selective uptake using two strains, one unable to consume glucose and one unable to consume xylose. The xylose-selective strain ALS998 has mutations in the three genes involved in glucose uptake, rendering it unable to consume glucose: *ptsG* codes for the Enzyme $IICB^{Glc}$ of the phosphotransferase system (PTS) for carbohydrate transport (Postma et al., 1993), *manZ* codes for the IID^{Man} domain of the mannose PTS permease (Huber, 1996), *glk* codes for glucokinase (Curtis and Epstein 1975) We also constructed strain ALS1008 which has a knockout in the *xylA* gene encoding for xylose isomerase, rendering ALS1008 unable to consume xylose.

Two batch experiments and one continuous bioprocess were completed. In the first experiment, each strain was grown *separately* in a defined medium of 8 g/L xylose and 15 g/L glucose which represented xylose and glucose concentrations that can be generated by actual biomass. In the second experiment, the two strains were grown *together* in batch in the same defined, mixed-sugar medium. In a third experiment, we grew the strains continuously in a "chemostat", except that we shifted the concentrations of glucose and xylose periodically to observe how the system would respond. (For example, we shifted the glucose concentration suddenly from 15 g/L to 30 g/L in the feed).

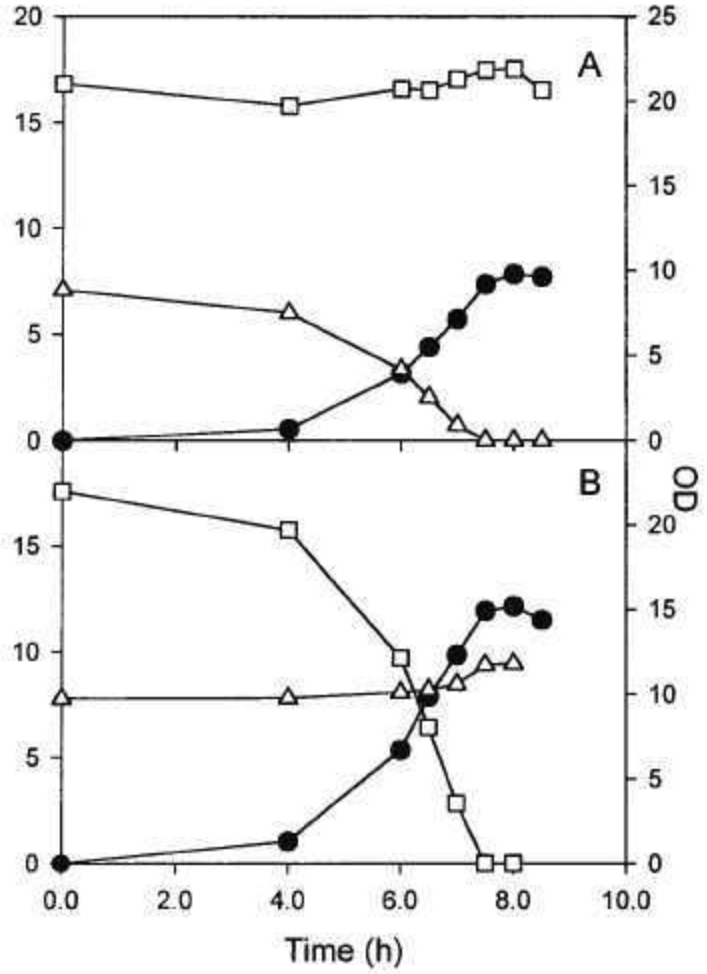

Figure 1: Batch aerobic fermentation of single *E. coli* strains on a mixture of glucose (□) and xylose (△). The OD (●) is measured over the course of the fermentation time. A) CGSC5457 only and B) ALS1008 only.

In the process containing only ALS998 (Figure 1A), 8 g/L xylose was completely consumed in 7 h, whereas glucose was never consumed. In the process containing only ALS1008 (Figure 1B), we observed the complete consumption of 15 g/L glucose in 7.5 h, while the concentration of xylose remained essentially unchanged. This experiment confirms that ALS998 consumes only xylose, and ALS1008 consumes only glucose. In the second experiment with ALS998 *and* ALS1008 (Figure 2), we inoculated both strains at the same initial cell density into one bioreactor containing 8 g/L xylose and 15 g/L glucose. For this co-culture process, glucose was consumed in 7.5 h, xylose was consumed in 7.0 h (Figure 2). Also, the culture reached a final optical density (OD) of about 25, which corresponds to the sum of the OD's observed for the two fermentations in the first experiment. Thus, each strain behaved independently in its growth and substrate consumption. ALS998 ignored glucose and ALS1008 ignored xylose. (Importantly, ALS998 consumed xylose as quickly alone as it did in the presence of the other strain.)

We next completed a "chemostat" using both strains simultaneously, except that we shifted the concentrations of xylose and glucose (up or down between 5-40 g/L) several times over the course of a 50 hour period of pseudo-steady-state. Because one of the strains (ALS1008) was resistant to tetracycline, we also were able to determine the population of each strain in this mixed culture. Despite shifting the concentrations of both glucose and xylose, the

concentration of each in the effluent was zero throughout the experiment. Moreover, the population of each strain adjusted to the feed composition. For example, when the xylose concentration of the feed increased, the population of the xylose-selective strain increased in response to that shift. This observation confirms that each strain is behaving independently, and

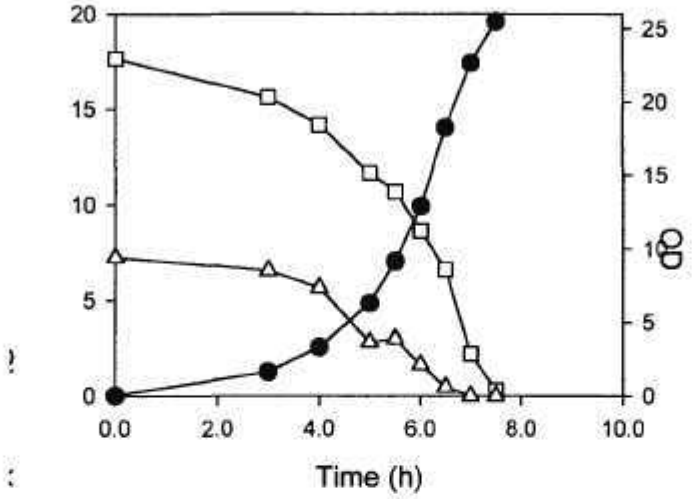

Figure 2: Batch aerobic co-fermentation of *Escherichia coli* strains ALS998 and ALS1008 on a mixture of glucose (□) and xylose (△). The OD (●) is measured over the course of the fermentation time.

is ignoring the carbon-source that it cannot consume. As long as the feed composition was carbon-limited, both substrates were consumed at the rate they were supplied. From these preliminary experiments we have established that xylose and glucose can be consumed simultaneously and that the system is self-adapting to varying concentrations of these two substrates. This is a phenomenal advantage of the proposed strategy.

Having demonstrated that xylose-selective and glucose-selective strains can be used together in a single process to consume these two sugars, we next focused on the second question: Can these strains be used to accumulate a product simultaneously from xylose and glucose? We selected lactate because it is straightforward for *E. coli* to accumulate this model compound. Although we have constructed these strains to consume xylose and glucose, these strains did not have any genetic modifications to enhance the formation of lactate (or any other product). A mutation in *pflB* encoding for pyruvate formate lyase causes a severe metabolic bottleneck at pyruvate under anaerobic conditions, curtailing growth in the absence of acetate and diverting most carbon to lactate (de Graef et al. 1999). Therefore, we knocked out the *pflB* gene in each strain to form ALS1073 (ALS998 *pflB*) and ALS1074 (ALS1008 *pflB*). ALS1073 would be expected to consume only glucose, while ALS1074 only xylose, in a mixture of glucose and xylose. Because of the one additional mutation, both strains should accumulate lactate under non-growth anaerobic conditions.

We completed three experiments to focus on lactate generation from xylose-glucose (20 g/L:30 g/L) mixtures: ALS1073 alone, ALS1074 alone, and the two strains together. In order to

generate lactate, we grew the strains aerobically for 8 h, and then switched to a non-growth anaerobic phase.

In our first experiment with ALS1073 alone, this strain consumed approximately 10 g/L glucose during the 8 h of growth to an OD of 11.5. After commencing anaerobic conditions, the remaining 17 g/L glucose was converted into about 14 g/L lactate within 3 h for a yield of 0.83 g/g. About 1.5 g/L succinate and less than 0.5 g/L acetate and ethanol as by-products were also generated during the anaerobic phase. Lactate was formed at a constant specific rate of formation of about 1.2 g/g·h during the anaerobic phase. During the two phases of growth and product formation, the xylose concentration remained unchanged.

In our second experiment, ALS1074 consumed 4 g/L xylose during the 8 h aerobic growth phase to an OD of 4.5. During the anaerobic phase, the remaining 13.3 g/L xylose was converted to 13 g/L lactate for a yield near 1.0 g/g. The conversion of xylose to lactate was completed after 14 h. The rate of xylose consumption by ALS1074 appears slower than

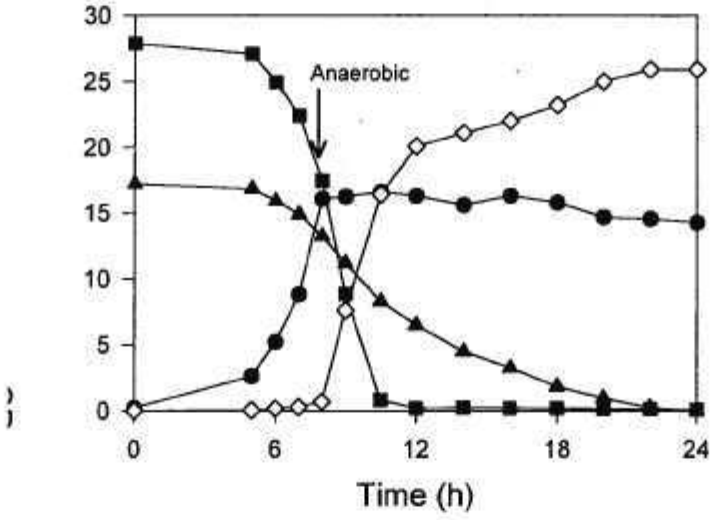

Figure 3: Batch aerobic-anaerobic process of *Escherichia coli* strains ALS1073 and ALS1074 on a mixture of glucose (■) and xylose (▲). After 8 h of aerobic growth, the culture was switched to anaerobic conditions. The OD (●) and lactate concentration (◇) were measured over the course of the co-fermentation.

observed for the rate of glucose consumption by ALS1073. However, this observation must be considered in light of the significant difference in cell biomass concentration between the two experiments—the cell concentration for the xylose-only consumption using ALS1074 was 40% of the concentration for glucose-only consumption using ALS1073. The specific rate of xylose consumption was 0.92 g/g·h at the onset of the anaerobic phase. Less than 0.5 g/L succinate and no acetate and ethanol were generated as by-products, and the glucose concentration remained unchanged during the entire process.

To demonstrate simultaneous xylose and glucose consumption to form lactate, *both* ALS1073 and ALS1074 were inoculated into a single bioreactor containing the xylose-glucose

defined medium. In this two-strain co-fermentation, care was taken to ensure that the inoculum consisted of each microorganism at the same concentration as had been inoculated in the one strain processes. As shown in Figure 3, after 8 h of growth, the culture had consumed about 4 g/L xylose and 10 g/L glucose to achieve an OD of 16. During the subsequent anaerobic phase, the remaining glucose was consumed in less than 3 h, and the xylose was consumed in about 12 h. Assuming that the OD of 16 represents an OD of 11.5 for ALS1073 and an OD of 4.5 for ALS1074 (values observed in the previous single organism cases), then the specific rate of glucose consumption during the anaerobic phase was 1.6 g/g·h, and the rate of xylose consumption was initially 1.2 g/g·h. The 17.5 g/L glucose and 13.3 g/L xylose present at the onset of the anaerobic phase were converted to 25.9 g/L lactate, for a combined yield of 0.84 g/g. About 2 g/L succinate and less than 0.5 g/L acetate and ethanol were generated as by-products. The experiment dramatically demonstrates that the two strains acted independently in the conversion of xylose and glucose to lactate.

Although the two-strain process as implemented (Figure 3) performed exactly as each single strain process, the two-strain process exposes one shortcoming which can uniquely be overcome with the multi-strain approach. Under the conditions of the experiment, the volumetric rate of xylose consumption did not match the volumetric rate of glucose consumption. Specifically, because glucose exhaustion occurred in less than 3 h of anaerobic conditions but xylose consumption required over 12 hours, the process inefficiently consumed only one of two possible substrates for the final 10 h. The overall process was essentially limited by the volumetric rate of xylose consumption. To maximize overall productivity, the two consumption rates ideally would allow both glucose and xylose to be exhausted at the same time. In a one-strain approach for the simultaneous consumption of xylose and glucose, only one biomass concentration exists, and the process does not have the flexilibity of adjusting volumetric consumption rates. However, in a multi-strain process, not only can each strain's specific consumption rate be altered (through metabolic engineering strategies), but each strain's biomass concentration can be independently controlled. This additional degree of control permits unrivaled process design flexibility.

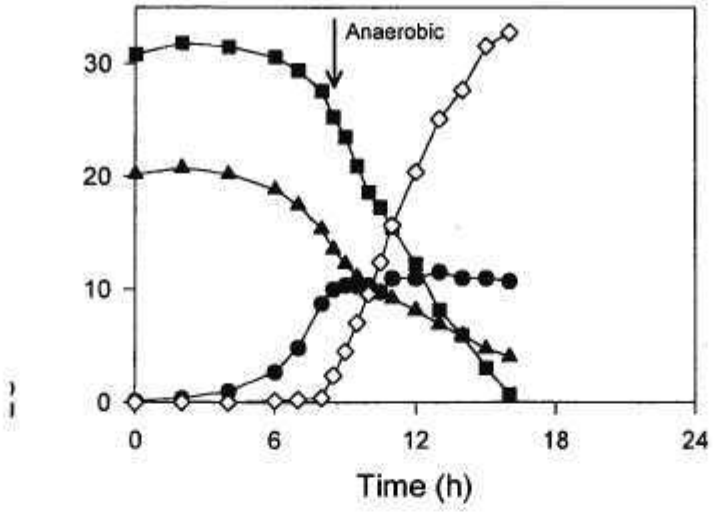

Figure 4: Batch aerobic-anaerobic process of two *Escherichia coli* strains ALS1073 and ALS1074 on a mixture of glucose (■) and xylose (▲). At the start of the process the bioreactor was inoculated with ALS1074, and after two hours the bioreactor was inoculated with ALS1073. After 8.5 h of aerobic growth, the culture was switched to anaerobic conditions as indicated. The OD (●) and lactate concentration (◇) were measured over the course of the co-fermentation.

We increased the cell density of the xylose-consuming strain exclusively by providing this strain more time for growth prior to switching to the non-growth production phase. Specifically, we again used a medium with 20 g/L xylose and 30 g/L glucose; however, at the start of the process (t=0), the bioreactor was only inoculated with the xylose-consuming strain ALS1074. Two hours after this inoculation, the bioreactor was inoculated with the glucose-consuming strain ALS1073, and at 8.5 h, anaerobic conditions commenced. Therefore, the xylose-consuming strain experienced 8.5 h of aerobic growth, while the glucose-consuming strain was experienced only 6.5 h of aerobic growth. At the time that anaerobic conditions commenced, the OD of the culture was approximately 10.5 (Figure 4), and we estimate that about 60% of the biomass was ALS1074 while 40% of the biomass was ALS1073. In this final experiment, the rates of glucose and xylose consumption were much more closely matched with both sugars nearly exhausted simultaneously. Thus, the two-sugar mixture was efficiently converted at a constant rate into 32 g/L lactate over the course of 8 h. The small sacrifice made in the unnecessarily large glucose consumption rate was more than offset by the improvement in xylose-consumption rate.

To demonstrate that the substrate-selective approach could be used to generate ethanol, we had hoped to be able to use the widely utilized ethanol-generating plasmid pLOI295. We transformed the pLOI295 plasmid into ALS1073 and ALS1074, because the *pflB* knockout is also required to produce ethanol. Unfortunately, we found that the pLOI295 and pLOI297 plasmids were very unstable and produced much lower amounts of ethanol than expected. Because other researchers have encountered problems with the pLOI295 and pLOI297 plasmids (Hespell et al., 1996; Lawford and Rousseau, 1996) and both of these plasmids utilize pUC backbones which are known to be problematic, we decided to reclone the *Z. mobilis pdc* and *adhB* genes from pLOI295 into pTrc99A using an optimized Shine-Dalgarno ribosome binding

site to enhance the translation of the pET genes in E. coli. The resulting plasmid, pTrc99A-*pet* produced significantly more ethanol than the pLOI295 and proved to be very stable.

ALS1073 and ALS1074 containing pTrc99A-*pet* were grown in a mixture of 10 g/L xylose and 14 g/L glucose. We were surprised that the strains generated only some ethanol, but instead accumulated a significant quantity of lactate. With respect to the ability to consume the xylose and the glucose simultaneously the system behaved as expected. In order reduce the lactate formation, we lowered the temperature to 30°C and repeated a set of experiments. The experiments were analogous to what we accomplished for lactate production: each strain alone, and one experiment with the two strains together in two-phase aerobic-anaerobic processes. For the ALS1073/pTrc99A-*pet* alone, the process generated 2.9 g/L ethanol only from glucose (i.e., xylose remained). For ALS1074/pTrc99A-*pet* alone, 2.3 g/L ethanol was generated from xylose with no glucose consumption. For the mixture of the two strains, 5.7 g/L ethanol was generated. This experiment was not optimized for ethanol production, but the results clearly demonstrate the ability of this approach to consume mixtures of sugars simultaneously for the production of products such as ethanol or lactate.

The third question we asked as part of our preliminary studies is whether this strategy could be used to consume the inhibitor acetic acid selectively from a mixture also containing sugars xylose and glucose. Surprisingly, *E. coli* readily consumes acetic acid as a sole carbon/energy source (El-Mansi et al., 2006), but it generally will not consume acetate in the presence of other substrates from which the cells can derive more energy. Thus, the presence of acetate diminishes the consumption rates of other substrates, such as glucose and xylose. However, we reasoned that *E. coli* can be *forced* to grow on acetate and prevented from consuming glucose or xylose (for example) by knocking out the genes which encode for glucose *and* xylose consumption. We refer to such a strain as "acetate-selective" because of the three substrates, acetate is its exclusive carbon nutrient.

We therefore tested whether acetate could be selectively removed from a mixture of xylose, glucose and acetate. We used *E. coli* MG1655 to generate ALS1060. MG1655 is a common wild-type strain (Jensen, 1993), and we verified that it grows aerobically with acetate as the sole carbon source at a growth rate of approximately 0.24 h^{-1}. ALS1060 has four knockouts of genes coding for proteins involved in xylose and glucose utilization (these genes were described above): *ptsG*, *manZ*, *glk*, and *xylA*. These four mutations should prevent the consumption of either xylose or glucose by ALS1060, but have no known affect on acetate metabolism.

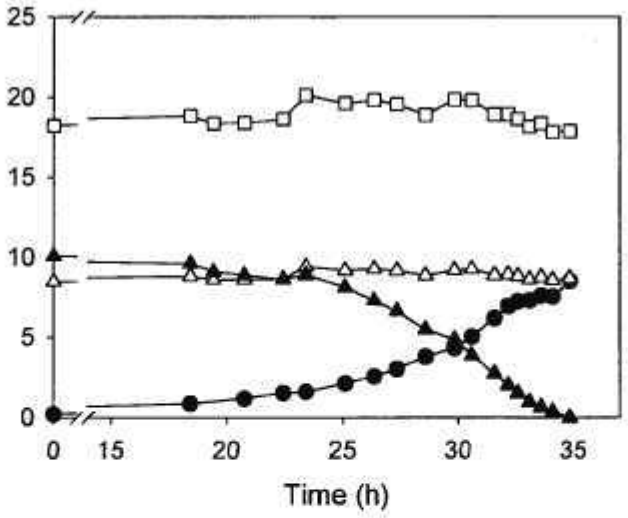

Figure 5: Batch aerobic fermentation of *Escherichia coli* ALS1060 (knockouts in *ptsG manZ glk xylA*) on a mixture of glucose (□), xylose (△) and acetate (▲). The OD (●) was measured over the course of the fermentation time.

We grew ALS1060 in a batch process using a medium containing an extremely high proportion of acetate: 10 g/L acetate, 10 g/L xylose and 20 g/L glucose. In this case, ALS1060 consumed 10 g/L acetate at a growth rate of 0.16 h^{-1}, but left xylose and glucose unconsumed even after 40 hours (Figure 5). The significant lag phase observed in this experiment can be attributed to using the poor MG1655 strain and the sudden exposure to 10 g/L acetate. Acetate consumption depends on the cellular balance between the glyoxylate shunt and isocitrate dehydrogenase (Holms, 1986), and different *E. coli* strains would be expected to behave quite differently. Our selection of MG1655 was merely to provide evidence that the concept would work; this strain turned out not to be a good acetate-grower. We have recently found *E. coli* strains which consume acetate at a growth rate over 0.70 h^{-1} with no initial lag in growth, and will use these strains to develop the acetate-selective strain as described in the Work Plan.

In these preliminary experiments we have been able to 1) remove acetate selectively from a mixture of xylose, glucose and acetate and 2) consume xylose and glucose simultaneously in a mixture of these two sugars, 3) generate lactate efficiently from synthetic mixtures of sugars. These steps can be linked together in a *two-stage* process to generate a product like ethanol, as conceptualized in Figure 6 from an acetate/xylose/glucose mixture. After removal of acetate in Stage 1, the remaining mixture is subsequently fermented in a second process stage to the desired product. Because knock-out strains are very stable, the strategy can readily be extended to any number of substrates. For example, an arabinose-selective strain will be unable to consume xylose and glucose, etc. The first stage could use one species of organism such as *E. coli*, while Stage 2 could involve another species such as either *E. coli* or *S. cerevisiae*.

Rather than try to develop a single organism to accomplish all the process design goals required for lignocellulosic conversion, our novel approach uses multiple strains to do tasks efficiently and independently. Note that no *competition* exists in the envisioned co-culture (in Stage 2). Competition involves multiple species competing for the *same* substrate. In this case, the strains each seek only their specific substrate and, being otherwise the same, do not interfere with each other. Thus, potential shortcomings from competition in a "mixed-culture" bioprocess are avoided.

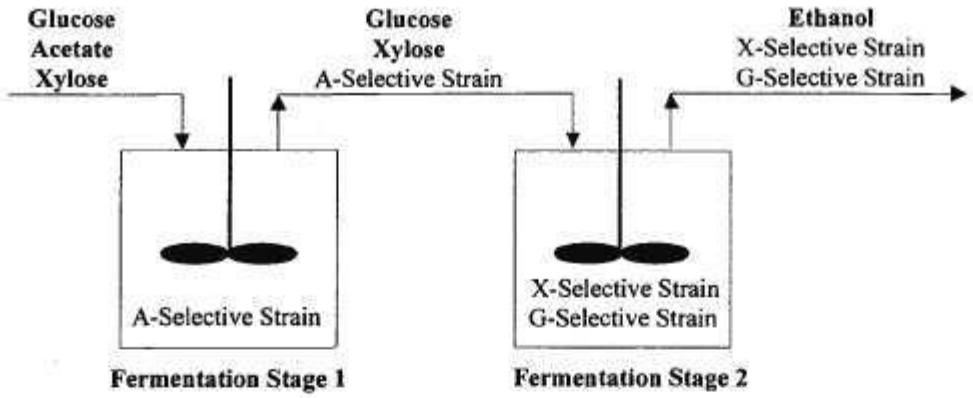

Figure 6: Envisioned Two-Step Process to 1) Consume inhibitor acetic acid and 2) to convert xylose and glucose simultaneously into a desired product.

There are several significant advantages that the envisioned continuous/fed-batch process has for the elimination of acetic acid and the simultaneous conversion of sugar mixtures, as exemplified by xylose and glucose:

1) Because the acetate-selective strain cannot grow in the absence of acetic acid, these cells will ultimately lyse in the second stage. By lysing, the cellular nutrients derived from acetate utilization are available to support growth of cells present in Stage 2. So, the inhibitor actually *enhances* product formation by enriching the hydrolysate with growth nutrients. It does not matter if the cells consume additional nutrients such as nitrogen, phosphorus, sulfur. These nutrients are conserved and become readily available to the organisms in the second stage. The goal of the first stage is only to consume acetate (quickly) and not consume any sugars.
2) Metabolic engineering strategies can focus on improving the individual production strains independently. For example, additional work can be devoted to improving the glucose-selective strain for ethanol production without concern for the impact of these changes on the conversion of xylose or on acetate tolerance/degradation. We do not need to compromise one objective for another.
3) The system adapts to fluctuations in the feed stream; that is, cultures actually grow in concert with the feed composition. For example, the system would respond to an increase in acetate concentration merely by increasing the cell density in Stage 1. Regardless of the perturbation of acetate in the feed (within a large range, as long as the cells remain carbon limited), this inhibitor will be completely removed in Stage 1. Similarly, as we have already observed, the system responds to an increase in the feed xylose concentration by increasing the cell density of the xylose-selective strain in Stage 2 (with no change in the cell density of any other strain). Using a fed-batch process would prevent sugar accumulation, and each strain would convert its target sugar at high yield and productivity. *Operational robustness* is the hallmark of this process strategy, and it constitutes a major advance toward the utilization of lignocellulosic biomass.

3. Future Studies

The next step in the evolution of this technology is to develop and demonstrate at the pilot scale (100L) a substrate-selective uptake strategy for utilizing lignocellulosic hydrolysates. The organisms proposed to develop this process are *Escherichia coli* and *Saccharomyces cerevisiae*. We have already demonstrated that the concept of substrate-selective uptake works. Specifically, using *E. coli* we have demonstrated that a xylose-selective strain will consume only xylose and a glucose-selective strain consumes only glucose in a mixture of these two sugars and effectively accumulate lactate. We have also demonstrated that a key inhibitor, acetate, can be selectively removed from a mixture of acetate, glucose and xylose.

The strains used for our preliminary studies were not derived from optimally-growing strains. Better strains exist for consuming lignocellulosic hydrolysate and generating ethanol, and the process can be optimized. Generally, the iterative approach will be to construct strains based on gene deletion and/or heterologous gene transformation, and then examine their behavior in controlled bioreactors. Although much of the work will occur in parallel or will overlap, *for the sake of clarity*, the specific aims are:

a. Develop *E. coli* strains which selectively use sugars
b. Introduce the ethanol pathways into the strains
c. Develop *S. cerevisiae* strains which selectively use sugars
d. Develop fermentation process using simulated sugar mixtures
e. Develop process to consume acetate without sugar degradation
f. Study at 100 L scale the entire process using simulated and real hydrolysates

We plan to develop a process using *E. coli* for the selective removal of acetate (Stage 1 in Figure 6), while we plan to develop processes using *E. coli* and *S. cerevisiae* for the simultaneous conversion of mixed sugars (Stage 2 in Figure 6). While we intend to have a mixture of strains from only one species of organism (*E. coli* or *S. cerevisiae*) in Stage 2, it is possible that processes could be developed which combine substrate selective *S. cerevisiae* strains with substrate selective *E. coli* strains in Stage 2.

4. Implementation and Project Management Plan

a. Develop E. coli strains which selectively use sugars

This part of the project involves constructing (five) different strains which together will consume the (five) principal sugars. Before we detail the planned research activities, we describe the genes specific to the uptake of each of the five sugars present in lignocellulosic hydrolysates. Table 1 summarizes the genes involved in uptake of the five sugars arabinose, galactose, glucose, mannose, and xylose.

Glucose: Like many sugars, glucose must first be phosphorylated before it can be further metabolized by *E. coli*. The principal route to phosphorylate glucose is by the phosphotransferase system (PTS). Because the PTS phosphorylates several sugars, some of the enzymes involved have broad specificities and can phosphorylate more than one sugar (Postma et al., 1993). Glucose can be phosphorylated by two different enzymes of the PTS,

glucosephosphotransferase and mannosephosphotransferase encoded respectively by the *ptsG* and *manZ* genes (originally designated as *gpt* and *mpt*). Furthermore, glucose can be phosphorylated by the enzyme glucokinase which is encoded by the *glk* gene (Curtis and Epstein, 1975). Glucose auxotrophic strains will be constructed by knocking out the genes encoding for glucosephosphotransferase (*ptsG*), mannosephosphotransferase (*manZ*) and glucokinase (*glk*).

Xylose: Xylose is transported into *E. coli* by the xylose transport system and once internalized must be isomerized to xylulose by xylose isomerase and phosphorylated to xylulose-phosphate by xylose kinase before it can be metabolized (David and Weismeyer, 1970). Xylose auxotrophic strains will be constructed by knocking out the gene encoding for xylose isomerase (*xylA*).

Arabinose: Arabinose is another sugar found in biomass hydrolysates, accounting for about 5% of the total sugar. The consumption of arabinose into *E. coli* is essentially analogous to the xylose uptake process. Arabinose consumption can be eliminated by knocking out the *araA* gene.

Galactose: The hexose galactose is a component in lignocellulosic hydrolysate, but is commonly found at concentrations of 1% or less (Lee, 1997). Galactose uptake is mediated by galactokinase encoded by the *galK* gene, and galactose uptake is eliminated in a *galK* mutant.

Table 1: Pathways for sugar utilization in *E. coli*

Enzyme	Gene	Reaction catalyzed
Arabinose Pathway		
Arabinose-Binding Protein	*araF*	Arabinose transport
Arabinose Transport Membrane Protein	*araH*	Arabinose transport
Arabinose ATPase Protein	*araG*	Arabinose transport
Araninose Isomerase	*araA*	Arabinose → Ribulose
Ribulokinase	*araB*	Ribulose → Ribulose-5-P
Galactose Pathway		
Galactose Binding Protein	*mglB*	Galactose transport
Galactose Transport Membrane Protein	*mglC*	Galactose transport
Galactose ATPase Protein	*mglA*	Galactose transport
Galactokinase	*galK*	Galactose → Galactose-1-P
Glucose Pathway		
Glucokinase	*glk*	Glucose → Glucose-6-P
Glucosephosphotransferase Enzyme II	*ptsG*	Glucose → Glucose-6-P
Mannose PTS Protein IIA(III)	*manX*	Glucose → Glucose-6-P
Pel Protein	*manY*	Glucose → Glucose-6-P
Mannosephosphotransferase Enzyme IIB	*manZ*	Glucose → Glucose-6-P
Mannose Pathway		
Mannose PTS Protein IIA(III)	*manX*	Mannose → Mannose-6-P
Pel Protein	*manY*	Mannose → Mannose-6-P
Mannosephosphotransferase Enzyme IIB	*manZ*	Mannose → Mannose-6-P
Xylose Pathway		
Xylose Proton Symport Protein	*xylE*	Xylose transport
Xylose Isomerase	*xylA*	Xylose → Xylulose
Xylulokinase	*xylB*	Xylulose → Xylulose-5-P

Mannose: The presence of the hexose mannose in lignocellulosic hydrolysate varies widely between 0-12% depending on the biomass (Lee, 1997). Mannose is phosphorylated exclusively by the mannose-specific components of the PTS (e.g., mannosephosphotransferase) encoded by *manX*, *manY* and *manZ*. Preventing glucose uptake by the triple mutations as described above (ptsG, glk, *manZ*) will simultaneously prevent mannose consumption. Similarly, knocking out *xylA* and *araA* (and *galK*) will have no impact on mannose consumption. By deleting only *manX*, *manY*, or *manZ*, a strain can be constructed that will not consume mannose, but still consume glucose at wild-type rates (Curtis and Epstein, 1975). We cannot construct an *E. coli* strain which consumes mannose but not glucose. Fortunately, the conversion of mannose to ethanol does not interfere with the conversion of glucose to ethanol, and this strain is unnecessary.

In order to consume all the sugars present in lignocellulosic hydrolysates simultaneously, strains will be constructed which consume each one of these sugars (alone). Thus, the following strains will be constructed:

1) glucose-selective strain: mutations in *araA galK xylA (manZ)*
2) xylose-selective strain: mutations in *ptsG manZ glk araA galK*
3) arabinose-selective strain: mutations in *ptsG manZ glk xylA galK*
4) galactose-selective strain: mutations in *ptsG manZ glk araA xylA*
5) mannose-selective strain: *araA galK xylA*

It should be noted that any single knockout requires about two to three weeks of full-time effort, however, multiple strains can be developed at the same time. Thus, constructing xylose-selectivity (5 genes) in a desired host strain will require about three months, and several other strains can be developed simultaneously. As a comparison, the commercial strain of *E. coli* used for 1,2-propanediol production contains 18 mutations as well as numerous heterologous genes (Sanford et al., 2004).

Our preliminary experiments were conducted in *E. coli* K12 or its non-isogenic derivatives (see Introduction), and included xylose and glucose, not the two other uniquely consumed sugars galactose and arabinose. The construction of any substrate-selective strain requires knocking out all other uptake systems. So, for example, to construct a xylose-selective strain, we will knock out the *glk*, *ptsG*, *manZ*, *galK* and *araA* genes in the *E. coli* B strain. The B strain has been chosen for this project because it is a widely used prototrophic derivative of wild-type *E. coli* that is well characterized and grows rapidly in both defined and complex media. Furthermore, our experience is that this strain grows very well on a variety of carbon sources (e.g., xylose, arabinose, etc.). Other research groups have also favored derivatives of the B strain (Ingram et al., 1999; Tao et al., 2001). The *E. coli* gene knockouts will be constructed using the Keio collection of single-gene knockout mutants (Baba et al., 2006). If necessary, the lambda Red recombination system (Yu et al., 2000; Datsenko and Wanner, 2000) will also be employed to construct the required gene knockouts. We have a lot of experience using these approaches. Where possible for control purposes we will compare substrate-selective strains that we construct to similar publicly available strains. The four substrate-selective B strains (one for each of glucose, xylose, arabinose and galactose) will be useful platforms for other researchers to develop processes for the formation of various products from the five sugars (including mannose).

Additionally, using the same approach we will construction four substrate-selective using the ethanologenic *E. coli* strain "KO11". The result of this portion of the research will be eight strains (four derived each from B and KO11).

b. Introduce the ethanol pathways into the strains

In anticipation of generating ethanol, we will also have to knockout genes which involve pathways competing with ethanol formation. We anticipate significant lactate and formate will be generated for the B-derived strains listed above, so that further strain modification will be completed: deleting the *ldhA* gene which encodes lactate dehydrogenase will eliminate lactate from being produced while deleting the *pfl* gene which encodes pyruvate formate lyase will eliminate formate. We have used this strategy to increase the fermentation yields of several products in *E. coli* (Tomar et al., 2003; Lee et al., 2004; Smith et al., 2006; Zhu et al., 2007).

Decades of research have led to great improvements in strains of *E. coli* that accumulate ethanol, and the proposed approach can be adapted to current or future *E. coli* ethanologenic strains. The approach can also be adapted readily to strains of *E. coli* which generate any other compound. This portion of the project focuses on the simultaneous and efficient use of sugar mixtures, and it is desirable to use this approach towards the production of a specific compound, and we propose to study ethanol. At this phase of the research, we will have constructed various sugar-selective strains from KO11 and B. We will introduce the ethanol pathways into the B strains using pTrc99A-*pet*. The result of this portion of the research will be two sets of substrate-selective ethanologenic strains of *E. coli* (8 total).

c. Develop S. cerevisiae strains which selectively use sugars

The process to generate substrate-selective strains in *Saccharomyces cerevisae* will be analogous to the approach employed with *E. coli*. *S. cerevisiae* is the preferred microorganism in ethanol production as this yeast tolerates very high ethanol concentrations. The pathways for sugar utilization in yeast are shown in Table 2. Genetically modified *S. cerevisiae* strains have been isolated that can produce ethanol concentrations up to 18% with very high productivity. Unfortunately, the use of *S. cerevisiae* to produce fuel ethanol from lignocellulosic hydrolysates is problematic. *S. cerevisiae* cannot utilize the pentose sugars xylose and arabinose which are two of the most abundant sugars that are found in lignocellulosic hydrolysates, because it lacks several of the key enzymes required for arabinose and xylose utilization. Researchers have partly solved the problem for xylose utilization by constructing new *S. cerevisiae* derivatives that contain the genes required for xylose utilization (Ho et al., 1998: Toivari et al., 2001). These new *S. cerevisiae* derivatives can utilize xylose and produce ethanol. However, when these yeasts are fed a mixed carbon source that contains glucose and xylose, the glucose must be consumed first, before any xylose can be utilized (Kuyper et al. 2005).

Our multi-organism approach can be applied to *S. cerevisiae* as well. *S. cerevisiae* strains which can not utilize galactose can be constructed by deleting the *GAL2, GAL1, GAL7*, or *GAL10* genes. These strains will be able to utilize glucose as a carbon source, but not galactose, xylose, or arabinose. *S. cerevisiae* strains that cannot utilize glucose can be constructed by deleting the *GLK1, HXK1*, and *HXK2* genes (Maitra and Lobo, 1983) or the *HXT1, HXT2, HXT3, HXT4, HXT6, HXT7*, and *SNF3* genes (Liang and Gaber, 1996). These strains will be able to utilize galactose as a carbon source, but not glucose, xylose, or arabinose. As discussed above, *S. cerevisiae* strains which utilize xylose can be constructed by importing these pathways from another yeast that is able to utilize this sugar. By deleting the genes that are required for glucose and galactose utilization in these strains, new *S. cerevisiae* derivatives can be created which will only able to utilize xylose. Researchers have also shown that *S. cerevisiae* strains which can utilize arabinose to produce ethanol can be constructed by importing the missing enzymes from bacteria such as *E. coli, Bacillus subtilis*, or *Lactobacillus plantarum* (Becker and Boles, 2003; Wisselink et al., 2007). By deleting the genes that are required for glucose and galactose utilization in these strains, new *S. cerevisiae* derivatives can be created which will only able to utilize arabinose. We will construct *S. cerevisiae* strains that can selectively only consume glucose, galactose, xylose, or arabinose and demonstrate the effectiveness of our consortium approach to convert the mixed sugars from lignocellulosic hydrolysates into ethanol.

Table 2. Pathways for sugar utilization in yeast†

Enzyme	Gene	Reaction catalyzed
Arabinose Pathway		
Aldose Reductase	*GRE*	Arabinose → Arabinitol
Arabinitol-4-Dehydrogenase	*LAD1*	Arabinitol → Xylulose
Xylulose Reductase	*ALX1*	Xylulose → Xylitol
Xylitol Dehydrogenase	*XYL2*	Xylitol → Xylulose
Xylulokinase	*XKS1*	Xylulose → Xylulose-5-P
Galactose Pathway		
Galactose Permease	*GAL2*	Galactose transport
Galactokinase	*GAL1*	Galactose → Galactose-1-P
Galactose-1-P Uridyl Transferase	*GAL7*	Galactose-1-P → Glucose-1-P
UDP-Glucose-4-Epimerase	*GAL10*	UDP-Glucose → UDP-Galactose
Glucose Pathway		
Low-affinity glucose transporter	*HXT1*	Glucose transport
High-affinity glucose transporter	*HXT2*	Glucose transport
Low-affinity glucose transporter	*HXT3*	Glucose transport
High-affinity glucose transporter	*HXT4*	Glucose transport
High-affinity glucose transporter	*HXT6*	Glucose transport
High-affinity glucose transporter	*HXT7*	Glucose transport
Plasma membrane glucose sensor	*SNF3*	Glucose transport
Glucokinase	*GLK1*	Glucose → Glucose-6-P
Hexokinase isoenzyme 1	*HXK1*	Glucose → Glucose-6-P
Hexokinase isoenzyme 2	*HXK2*	Glucose → Glucose-6-P
Xylose Pathway		
Xylose Reductase	*XYL1*	Xylose → Xylitol
Xylitol Dehydrogenase	*XYL2*	Xylitol → Xylulose
Xylulokinase	*XKS1*	Xylulose → Xylulose-5-P

† xylose and arabinose pathways are not natively found in *S. cerevisiae*

d. Develop fermentation process using simulated sugar mixtures

A significant portion of this project will be devoted to the development and characterization of processes to metabolize sugar mixtures and ultimately in the presence of acetic acid (as described in a subsequent section). Initial studies will be performed on synthetic mixtures of 2-5 sugars (selected from the list of mannose, galactose, glucose, xylose and arabinose), while additional studies will be conducted on real hydrolysates supplemented with nutrients as required (e.g., nitrogen and phosphate sources ammonia and phosphate). These studies will use the *E. coli* and *S. cerevisiae* strains generated from previous sections, with 4 strains present in a co-culture simultaneously when all 5 sugars are present.

We will first need to establish the operating ranges for the processes through a series of chemostat experiments. While not generally used in industry, chemostat experiments provide an extremely useful way of determining the parameters necessary to design a relevant process (for example, a fed-batch process which is commonly used.) Like all fermentation experiments, these studies will involve highly-instrumented bioreactors in which feed-rate, temperature, pH, nutrient levels, oxygenation, etc. can be controlled. By studying a range of controlled growth

rates, we will determine for each strain the biomass yields, specific rates of consumption/production of dissolved and gaseous compounds, and the maintenance energy requirements resulting from the various genetic perturbations. Like any cells growing on different carbon-sources, these strains will have differing maximum growth rates. In a chemostat the microbial growth rate is determined by the nutrient feed rate (dilution rate), but the biomass concentration is determined by the limiting nutrient concentration. These maximum growth rates will help establish the maximum feed-rate of an envisioned fed-batch process. Operating a biological process at the maximum growth rate does not in general result in the maximum practical product formation rate for a variety of reasons such as oxygen requirement (for an aerobic process), heat duty, reduced biomass yield, genetic regulation of cells, etc. We will study the strains individually on single-substrate media, then single strains on multi-substrate media, then multiple strains on multi-substrate media. Drug resistances introduced into some strains will serve as selective markers to permit us to quantify the fraction of each strain comprising the total population. Questions that will be addressed include: does the presence of the unutilized sugar in any way impact the ability of the strain to consume its presumably exclusive substrate and generate ethanol, and what are the operating optimal conditions and ranges for the conversion process. In addition to the principal carbohydrates, major and minor fermentation products will be analyzed.

The chemostat experiments will provide us with parameters to enable the study and implementation of fed-batch processes. Our current concept is to feed in the mixed-sugar stream in a carbon-limited fashion (that is, each one of the four strains will be carbon limited for their sugar substrate). During an initial process "phase" cells will grow aerobically, while in a subsequent production phase, reduced oxygen availability will direct most of the carbon to the product ethanol. As long as the carbon-limited feed rate is lower than the capacity for carbohydrate uptake, the cells should respond to changes in the concentration of either substrate merely by increasing the biomass as we have observed in preliminary studies. We will confirm this expectation by introducing a temporally varying stream of mixed sugar into the fermenter, and monitoring how the composition of the culture changes (including the population of each strain). The result of this portion of the research will be a complete and quantitative description of the fermentation of sugar mixtures to ethanol by substrate-selective strains.

e. Develop process to consume acetate without sugar degradation

The goal of this portion of the research is constructing an *E. coli* strain which consumes acetate but not any of the five principal sugars in biomass hydrolysate as detailed above, and this process will require knockouts of all the sugar uptake systems. We have already demonstrated in preliminary experiments that this approach works for xylose and glucose, the two principal sugars in most lignocellulosic hydrolysates.

The first question we must address is what strain to use to make these knockouts. What strain has high growth rate and high biomass yield on acetate? Tolerance of the organism to high acetate concentration is not important (within a range) because we envision the process operating in fed-batch or continuous mode under carbon (i.e., acetate) limitation. Under these circumstances the concentration of acetate in the fermenter will be maintained at zero and cell "tolerance" to the acetate will not be relevant. However, the rate at which acetate is consumed will directly affect the productivity of the entire process. Therefore, the cells should ideally be able to grow at a high growth rate which will enable the process to run at a high (dilution) rate without any negative consequences.

Although several research groups have studied growth of *E. coli* on acetate and completed detailed flux analyses, there has not been a comprehensive comparison of the growth rate of *E. coli* strains on acetate as the sole carbon source. A quick examination of a couple strains during Phase I research demonstrated to us large differences in the maximum specific growth rate of various *E. coli* strains on acetate. (one strain achieved 0.70 h^{-1}, about 3 times faster than on MG1655 which was selected for the acetate-selective experiments shown in Figure 5). We plan a more comprehensive examination of about 8-10 diverse wild-type and common strains of *E. coli* (e.g., MG1655, DH5α, MC4100, BL21, JM109, etc.) and grow them as accelerostats (Paalme et al., 1997). We are particularly interested in B strains because these strains appear to have an elevated expression of enzymes in the glyoxylate shunt (van de Walle and Shiloach, 1998; Phue and Shiloach, 1998), which is an important pathway for acetate metabolism. Using this approach we will readily establish biomass yield and maximum growth rate. We will select the two "best" to knockout the sugar-consuming abilities (described below). Note that the strain found to be the "best acetate consumer" will not necessarily be related to the strain ultimately found to be the "best sugar consumer/ethanol producer". A significant advantage of our process design is that these two strains can be selected independently.

We are also interested in learning *why* certain strains grow more quickly on acetate, and will complete a genome-wide microarray study to clarify this question. For four of the strains (two "fast growers" and two "slow" growers), we will take samples from our accelerostat experiments (which occurs at a pseudo-steady state) at three different growth rates (approx. 0.1, 0.2, 0.3 h^{-1}). We will conduct microarrays comparing expression at these growth rates (i.e., 0.3 vs. 0.1 and 0.2 vs. 0.1) and at the highest growth rate between the strains (strain 2 vs. strain 1, strain 3 vs. strain 1, strain 4 vs. strain 1). With (independent) triplicate experiments, this will involve 15 microarrays. This approach is similar to our previous study at 6 steady-state growth rates that established the regulatory importance of the *arcAB* regulatory network in acetate overflow metabolism (Vemuri et al., 2006).

The second step in the study of acetate consumption will be to construct the acetate-selective strain, which must therefore have deletions in all genes involved in the uptake of glucose, mannose, galactose, xylose and arabinose. In the two strains selected for their "best" acetate metabolism, the six genes *manZ*, *ptsG*, *glk*, *xylA*, *galK*, *araA* will be knocked out as previously described. None of these genes has any known relationship with acetate consumption, a process which is mediated by acetyl CoA synthase (*acs*), isocitrate lyase (*aceA*), malate synthase (*aceB*) and isocitrate dehydrogenase (*icdA*) (Holms, 1986).

f. Study at 100 L scale the entire process using simulated and real hydrolysates

Two-stage fermentations of simulated mixed xylose, glucose, mannose, arabinose, galactose and acetate solutions as well as actual hydrolysates will be conducted using the constructed *E. coli* strains. A "simulated" solution is merely a synthetic (and reproducible) medium prepared with purified components in appropriate proportions to represent a real hydrolysate. In other words, we will prepare a defined medium containing these six compounds as potential carbon sources. Concentrations of these compounds in actual hydrolysates vary considerably (Barbosa et al., 1992; Johansson et al., 2001; Taherzadeh et al., 2001; Brandberg et al., 2004), and we will examine a range of concentrations: 20–40 g/L glucose, 5–20 g/L xylose, 1–5 g/L galactose/mannose/arabinose and 2–8 g/L acetic acid. We propose to rely on "real" hydrolysates obtained from researchers at DOE-NREL. As necessary, the hydrolysate will be supplemented with other nutrients such as nitrogen, phosphorus and sulfur.

The initial studies will use simulated hydrolysates. The first stage in the process (the left bioreactor in Figure 4) involves the removal of acetic acid by feeding an acetate-containing hydrolysate into the reactor so that the organisms grow continuously and the acetate-free stream with cells exits the vessel continuously. Using the growth rate data obtained previously (see part 4), we will feed the acetate at a dilution rate below the maximum growth rate and scale the process accordingly. We will determine the ranges of acetate concentration in the hydrolysate that are acceptable, and the rates for which the process can be conducted. We will determine the long-term stability of the process. The acetate consumption rate determined from such data will be necessary to size a pilot/commercial process. When acetate is the limiting nutrient under aerobic conditions, we anticipate that the carbon in acetate will be converted either into cells or CO_2. We will nevertheless need to complete a detailed carbon balance to account for the utilization of carbon under various operating conditions. The formation of the potential metabolic products such as ethanol, lactate, formate, succinate, and fumarate in the detoxification step is not anticipated but will be determined in addition to the concentrations of the six substrates via chromatography (Eiteman and Chastain, 1997). Additionally, we will demonstrate the robustness of the process by "ramping" the concentration of acetate in the feed and observing the response of the microbial system to changing acetate concentration. We anticipate that the cells will naturally adapt to changing concentrations of acetate in the feed stream by increasing the biomass concentration, as we have observed in preliminary studies for the two-substrate system. For simulated hydrolysate we anticipate that the stream exiting Stage 1 will contain exclusively 5 sugars (and biomass) as carbon sources.

Stage 2 will be fed the stream exiting Stage 1. Three different bioprocess modes will be examined for Stage 2, including batch, linear fed-batch, and exponential fed-batch. A batch process will be conducted by fermenting a discrete portion of the (continuous) effluent from Stage 1. Such a process will require storing some of the Stage 1 effluent in a tank. A linear fed-batch will be conducted by synchronizing the Stage 1 effluent rate with the feeding rate to Stage 2. In this case, because the biomass concentration in Stage 2 is small the feed initially into Stage 2 will exceed the rate of carbohydrate consumption, and the cells will grow at their maximum growth rate. Later after the biomass concentration increases, the maximum growth rate will exceed the carbohydrate feed rate and Stage 2 will become carbon limited. An exponential fed-batch, accomplished via a programmable pump, will involve holding a portion of the Stage 1 effluent initially and gradually increasing the rate of feeding to match a desired cell growth rate. Exponential fed-batch processes (e.g., Smith et al., 2006) are advantageous to control growth and product formation rates carefully. The focus of this portion of the research will be to study the kinetics of sugar consumption subsequent to acetate removal. Process stability, mode of operation, robustness to varying feed compositions and sensitivity to inoculation approaches will all be addressed in this portion of the project.

We will study ethanol production in this process, using a single organism process with *E. coli* KO11 (without additional substrate-selectivity) as an experimental control. Although a couple of different ethanol-production approaches are possible depending on the specific strain, we envision a two-step process for Stage 2 wherein the first step is an aerobic growth phase using the two strains and substrates. A second step involves a potential anaerobic production phase wherein all the carbon is directed to the product of interest, ethanol, and growth is low. Growth can be slowed by several means including limiting the feed in another nutrient such as nitrogen (i.e., ammonium ion).

We propose the inclusion of DOE-NREL and other FFRDC contractors as support resources. Specifically, we propose to obtain lignocellulosic hydrolysates from these groups,

and determine the detoxification of that hydrolysate using the acetate-selective strain in the developed process. We are particularly interested in corn stover hydrolysate. We will seek any liquid hydrolysate rather than the solid form. As noted, "raw" hydrolysate may require supplementation with additional nutrients in order to permit growth of the acetate-selective strain in Stage 1 and the growth of the 4 sugar-selective strains in Stage 2. Several commercial processes use nutrient supplementation which does not negatively impact the cost of production (ammonia, phosphate). We will readily be able to determine whether any particular hydrolysate is, for example, phosphate-limited rather than carbon limited by determining the nutrient levels in the effluent stream. We anticipate that the feasible feed rates for both stages will be lower when lignocellulosic hydrolysate is used compared to simulated hydrolysate. The results from these studies will be critically compared with the results obtained using the simulated xylose, glucose and acetate solution. These results will provide important data for an ultimate fed-batch process to be implemented in future commercial applications. Within the limits of the budget and timeframe, we propose to examine any hydrolysate deemed appropriate by FFRDC contractors or technical liaisons at DOE Office of Biomass.

Initial work on the entire process will use bioreactors of 2-5 L scale. Once the parameters have been established, we will complete several runs with synthetic hydrolysate and with real hydrolysate at the 100L scale. At this demonstration scale, we will be able to assess the material handling aspects of this process.

5. Project Timetable

There will be seven team members for this project. Two faculty who have approximately 50 years combined expertise in fermentation technology, microbial physiology and metabolic engineering will direct the project. Two research staff who have 30+ years combined experience will be involved in training, chemical analysis, overseeing molecular biology and fermentation protocols, enzyme assays, purchasing, coordinating student activities and conduct research. Two graduate students and one research associate will also participate in this research. One staff member and one graduate student will focus on fermentation process development with *E. coli* (Tasks d, e, f). One staff member and one graduate student will focus on *E. coli* strain development (Tasks a, b). The research associate will focus on *S. cerevisiae* and process development using this organism (Tasks c, d). Many of the tasks are independent and can be conducted in parallel. The participants will be housed in the same labs and will interact informally and formally.

The project will require 12 quarters, January 1, 2009 – December 31, 2011. Tasks:

a. Develop *E. coli* strains which selectively use sugars. Quarters 1 – 5. Each of the eight strains to be developed constitutes a milestone for the project.

b. Introduce the ethanol pathways into the *E. coli* strains. Quarter 5. Each of the eight strains into which the ethanol-generating plasmid will be transformed constitutes a milestone for the project.

c. Develop *S. cerevisiae* strains which selectively use sugars Quarter 1 – 8. This task will occur in parallel with Tasks a. and b., and is independent of these tasks. Each of the four strains to be developed constitutes a milestone for the project.

d. Develop fermentation process using simulated sugar mixtures. Quarter 4 – 10. This task will be the bulk of the effort.

e. Develop process to consume acetate without sugar degradation. Quarter 1 – 8. This task will occur in parallel with Tasks a. and b. The task involves developing an independent strain which consumes acetate. Once a strain is developed, a process will be developed using that strain in Stage 1 (see Figure 6) in conjunction with other strains from Tasks a., b. and c. in Stage 2.

f. Study at 100 L scale the entire process using simulated and real hydrolysates. Quarter 10 – 12. The fully developed proce4ss will be implemented on a larger scale and using various biomass hydrolysates.

6. Facilities

All facilities and intellectual resources required to complete the project tasks are available at the University of Georgia. The University of Georgia has established graduate level programs of research in renewable energy. These programs cut across both engineering, applied sciences and basic sciences. Prior federal funding from the Department of Energy (DE-FG36-01ID14007) has supported the development of coursework and infrastructure in engineering. Additional funding has been received to establish the Biorefining and Carbon Cycling Program and the Complex Carbohydrate Research Center as one of the DOE Bioenergy Research Centers. Strong administrative support exists as evidenced by the formation of a Bioenergy Program to unite and coordinate the vast campus activities.

The Center for Molecular BioEngineering (CMBE) where this research will take place occupies 6,000 sq. ft. and is located in the Driftmier Engineering complex. The Center is well equipped with the necessary equipment to carry out the molecular biology, fermentation, and analytical aspects of this project. Molecular biological facilities include microplate readers, dual-label scintillation counter, freeze drier, DNA gel electrophoresis (4), SDS-PAGE with western blot capability (4), DNA imaging system, speed-vac, fast ramp thermocycler (2), electroporator, UV crosslinker, chromatography refrigerator, dishwasher, autoclave, drying oven, cold room, automatic Petri-plate pouring machine, microfuges (4) and freezers (3). Fermentation facilities include 2.5 liter fermenters (9), 5.0 liter fermenter, 19 liter fermenter, incubators (8), biosafety cabinets (3), French pressure cell, centrifuges (3), crushed ice makers (2), anaerobic glove box, and water bath/shakers (7). Temperature, pH, and oxygen, carbon dioxide off-gas monitoring and control are used. Online glucose, lactate, glutamine and glutamate with feed-pump control are available. Chemical analysis facilities include gas chromatographs (3), spectrophotometers (3) and liquid chromatographs (4) with fluorometric, refractive index, UV/visible, and conductivity detectors. Portable GC/MS instruments (2) are available for gas or headspace analysis, and a dual column GC/MS with thermodesorption, cryofocussing and sniff port are also present. The University of Georgia is home to the Bioexpression and Fermentation Facility (http://www.uga.edu/bff/home.html). This facility houses multiple 5L fermenters, as well as

fermenters at the 24L, 100L, 200L, 400L, 500L and 800L scale. These bioreactors include oxygen supplementation, pH control, temperature control, process monitoring and continuous harvesting, batching. This resource is available at a nominal fee.

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