Final Technical Report

Project Title: ***A Novel simultaneous-Saccharification-Fermentation Strategy for Efficient Co-fermentation of C5 and C6 Sugars Using Native, Non-GMO Yeasts***

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Recipient: ***The University of Toledo***

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Final Scientific/Technical Report

1. Award identification

Award Number: *DE-FG36-08GO18163*
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Principal Investigators: *Sasidhar Varanasi & Patricia Relue*

2. Disclaimer

This report contains data and content from filed/issued patents and published journal articles.

3. Executive summary:

Economic bioethanol production is critically dependent upon the ability to convert both the hexose (C6) and pentose (C5) sugars resulting from cellulose and hemicellulose. C5 sugars are not readily fermentable by native *Saccharomyces cerevisiae*. Genetically Modified Organisms (GMOs) are designed to ferment xylose, but their stability, ethanol yield, environmental impact, and survival under conditions of industrial fermentation are unproven. In this project, we developed a novel approach for efficient fermentation of both C5 and C6 sugars using *native S. Cerevisiae* by exploiting its ability to produce ethanol from *xylulose* - the keto-isomer of xylose. While the isomerization of xylose to xylulose can be accomplished via commercially (and cheaply) available *Xylose Isomerase* (*XI*) (Sweetzyme™), this conversion has an extremely unfavorable equilibrium (xylose:xylose is about 5:1). To address this, we developed two alternate strategies. In the first, the two enzymes *XI* and urease are coimmobilized on solid support particles to enable complete isomerization of xylose to xylulose under pH conditions suitable for fermentation, in a *simultaneous-isomerization-fermentation* (SIF) mode. The ability of our technology to conduct isomerization of xylose under pH conditions suitable for both saccharification and fermentation opens the possibility of *SSF with native yeasts for the first time*. Herein, we performed specific research tasks for implementation of our technology in several modes of operation, including simultaneous-isomerization-and-fermentation (SIF), simultaneous-saccharification-and-isomerization (SSI) followed by fermentation, and SSF mode with the biomass feedstock poplar. The projected economics of our process are very favorable in comparison to the costs associated with engineering, licensing and propagating GMOs. This novel fermentation technology is readily accessible to rural farming economies for implementation in cellulosic ethanol production facilities.

4. Project accomplishments, goals, and objectives:

- *Project goals*

The goal of this project was to develop biocatalysts to facilitate utilization of xylose and C6 sugars by native yeasts for cellulosic ethanol production from biomass. This goal aligns well with DOE's Bioenergy Technology Office's objective of being able to efficiently utilize C5 sugars along with C6 sugars, as the former sugars constitute a significant portion of the total

sugars in lignocellulosic biomass. This goal is also aligned with two of the specific R&D objectives of the MYPP 2012 document: 1) technology development for producing fuels and bioproducts from lignocellulosic feedstocks; and 2) development of commercially viable technologies for converting biomass feedstocks via biochemical routes into energy-dense fungible liquid transportation fuels.

Economic bioethanol production from lignocellulose is critically dependent upon the ability to convert both the hexose (C6) and pentose (C5) sugars resulting from cellulose and hemicellulose. C5 sugars are not readily fermentable by native organisms. Genetically Modified Organisms (GMOs) are being investigated to ferment C6 and C5 sugars, but their stability, ethanol tolerance, environmental impact, and survival under conditions of industrial fermentation are unproven. Our approach involves isomerization of xylose to xylulose using immobilized *xylose isomerase* which can be fermented along with C6 sugars by native yeasts to produce ethanol from biomass hydrolysate with high yield. This approach will also be of great value from ethanol industry's perspective, as it allows seamless transition from corn ethanol to cellulosic ethanol. Three patents have been filed based on the technology developed during this project, of which one is issued and two are pending adjudication. A start-up company, SuGanit Systems Inc., has licensed these patents and is working on scale-up.

- *Objectives and accomplishments*

We proposed to develop biocatalysts for isomerization of C5 sugars, which are effective at pH conditions (pH 4-5) suitable for fermentation. This later requirement, as was explained more thoroughly in Section 5, necessitates co-immobilization of the enzymes *xylose isomerase* and *urease* within the catalyst pellet. Since saccharification of biomass also takes place at similar pH (pH 4-5), our technique can, in theory, be combined with the enzymatic saccharification of biomass and fermentation. Hence, simultaneous saccharification and fermentation (SSF) becomes feasible with native yeasts through the use of the biocatalyst pellets designed in this project. We identified the following specific tasks to accomplish the objectives for this project:

A - Biocatalyst development;
B - Design packed bed reactors for immobilized enzyme containment for use with filtered-hydrolysates;
C - Designs for immobilized enzyme pellet containment for solids-containing hydrolysates; and
D - Evaluation of alternative xylulose binding agents for use with the biocatalysts to enhance isomerization

A brief description of each task with milestones accomplished is given below. More detailed discussion of the relevance of these tasks to the project goals and results obtained can be found in Section 5.

<u>A Biocatalyst development</u>

Protocols for co-immobilization of xylose isomerase and urease were established to minimize enzyme cost. Two methods for producing the co-immobilized enzyme pellets were evaluated for robustness and effective lifetime in a packed-bed configuration employing simulated sugar mixtures as well as poplar hydrolysate prepared using dilute acid and ionic liquid pretreatments.

The milestone for this task was to optimize immobilized enzyme pellet design (XI:urease ratio) to achieve acceptable xylose isomerization (50% isomerized) @ 34°C. As documented in Section 5 (Figure 4), 90% isomerization of xylose to xylulose was achieved.

B Develop modular isomerization-followed-by-fermentation and simultaneous-isomerization-fermentation (SIF) configurations for use with filtered-hydrolysates

The goal of this task was to develop an SIF configuration that allows for recovery and repeated use of the biocatalyst pellets. We accomplished this using a packed bed of the co-immobilized enzyme pellets in combination with a hollow fiber membrane fermentor (HFMF) in a recirculation mode. We first designed, constructed, and operated the packed bed of enzyme pellets with the goal of maximizing xylulose production. Next, a hollow fiber membrane fermentor (HFMF), in which the yeast is confined to the shell-side and the filtered hydrolysate was passed through the fiber lumens, was employed to achieve sugar fermentation while keeping the product stream free of solids (i.e. cell mass). The HFMF design (1) provided efficient contact between sugars and yeast and (2) offered high cell densities needed for rapid pentose sugar fermentation. Finally, the packed-bed biocatalyst and HFMF modules were linked in a closed-loop to allow simultaneous-isomerization-and-fermentation (SIF). Ethanol yield and module regeneration times for SIF were measured and compared to sequential-isomerization-and-fermentation for efficiency of performance.

This task had five milestones.

The first milestone was to determine the number of runs for which the activity of the enzyme pellets in the packed bed retained at least 60% of their initial enzyme activity. We found no loss of enzyme activity of the immobilized pellets after more than 11 cycles of operation (Section 5 Figure 6).

The second milestone was to establish process conditions (temperature, pH, mass of pellets/liter of solution) needed to achieve acceptable xylulose yields (>70%) in the packed bed for translation to larger-scale operation. We were able to achieve this milestone by conducting Isomerization at T= 34 °C, pH 4.5 with 18 g enzyme pellets per liter of hydrolysates/sugar solution (Section 5, Figure 10)

The third milestone was to achieve ethanol yields in the HFMF that are comparable or better than traditional fermentor yields (0.35 - 0.45 g EtOH/g sugar) with model sugars; Section 5, Figure 11 shows that this milestone was successfully accomplished.

The fourth milestone was to obtain an ethanol yield in the HFMF for biomass hydrolysates that was comparable to that achieved with model sugar mixtures. This is demonstrated in Section 5, Figure 13.

The fifth milestone was a reduction in process time for SIF compared to fermentation using GMOs. We were able to ferment hydrolysates containing both C5 and C6 sugars to completion within 24 hrs (Section 5, Table 1).

C Develop a configuration that permits easy and rapid reuse of the co-immobilized enzymes for use with solids-containing hydrolysates

Strategies for immobilized enzyme use with solids-containing hydrolysates were successfully evaluated. This mode of operation allows both simultaneous saccharification and isomerization (SSI) as well as simultaneous saccharification, isomerization and fermentation (SSIF) (Section 5, Figures 7 & 8). However, as it was deemed that lignin recovery from the hydrolysates prior to fermentation was critical for process economics, the remainder of the project was focused on economic production and fermentation of isomerized, *filtered* hydrolysates (Task D).

D Evaluation of alternative xylulose binding agents for enhancing isomerization of xylose to xylulose with immobilized XI pellets and the recovery and reuse of these agents

Due to the inherent value of lignin, filtration of the hydrolyzate to recover lignin prior to further processing of the hydrolyzate is of commercial interest. Accordingly, Task D reflects aspects relevant to the economics of processes using filtered hydrolyzates. Recovery of the sugar complexing agents via their confinement to a phase immiscible with the hydrolysate significantly improved process economics through their reuse. In addition, any environmental concerns associated with their disposal have been eliminated.

This portion of the project had two milestones.

The first milestone was to establish concentrations of alternate xylulose binding agents that allow isomerization of xylose to xylulose of 60% or greater. The agents identified that satisfy this milestone are given in Section 5, Figure 15.

The second milestone was to establish if the sugar-binding agents reduced yeast growth, and if so, at what concentration of the agent yeast growth is reduced to 50% of the control cultures (no agent). Biocompatibility data for these agents is given in Section 5, Table 2.

In summary, this project has the potential to significantly increase the ethanol yield from lignocellulosic biomas by efficient utilization of xylose.. The approach we have implemented is to ***exogenously isomerize*** xylose to xylulose and ferment the resulting ketose sugar to ethanol using ***native*** yeast. By successfully executing all the proposed tasks and meeting all the project milestones, we have demonstrated

- a ***viable*** technology for fermenting C6 and C5 sugars to ethanol using (i) abundantly available *Xylose Isomerize* enzyme, (ii) agents that selectively bind to xylulose and (iii) **native** yeasts;
- how the readily-available immobilized *Xylose Isomerize pellets* and ketose-selective-agents lead to ***high yield*** conversion of xylose to xylulose, while simultaneously concentrating the ketose sugar;
- simple, robust, and scalable methods for producing immobilized biocatalyst and ketose-binding agent pellets, using commercially-available functionalized- supports;
- recovery and reuse strategies for the biocatalyst as well as the ketose-binding agent; and
- simultaneous saccharification and fermentation (***SSF***) via the addition of*XI* particles (with co-immobilizing urease) along with cellulases and native yeast to the pretreated-biomass.

- *Technoeconomic and other critical aspects relevant to the commercialization of the technology*

We have demonstrated the technical feasibility of the technology and have addressed several key elements that can significantly impact the technoeconomics of the process such as recovery and reuse of the catalysts, xylulose binding agents, and the yeast. However, a comprehensive technoeconomic analysis is outside the scope of this project. Nevertheless, we believe that the following aspects need to be critically evaluated in efforts aimed at commercialization.

– Added cost for xylulose production and fermentation must be less than the revenue generated from additional ethanol produced and must be competitive with the costs associated with xylose fermentation using GMO yeast/bacteria (licensing, propagation and nutrient cost)

– Since our technology is compatible with current corn ethanol techonology, ethanol-tolerant/adapted yeast strains from corn-ethanol industry can potentially provide an opportunity for increased sugar utilization and ethanol productivity. However, our technology should be evaluated with these strains.

– We have shown that our process (SIF) is compatible with both IL and dilute acid pretreatment. It is desirable to establish its broader applicability with hydrolyzates from other pretreatments and other feedstocks.

5. Summary of project activities

It is well known that, while *S. cerevisiae* can readily convert glucose from cellulose to ethanol, it cannot convert xylose from hemicellulose (see Figure 1). Over the past ten to fifteen years, lot of progress was made in genetic modification of both yeast and bacteria to impart them the ability

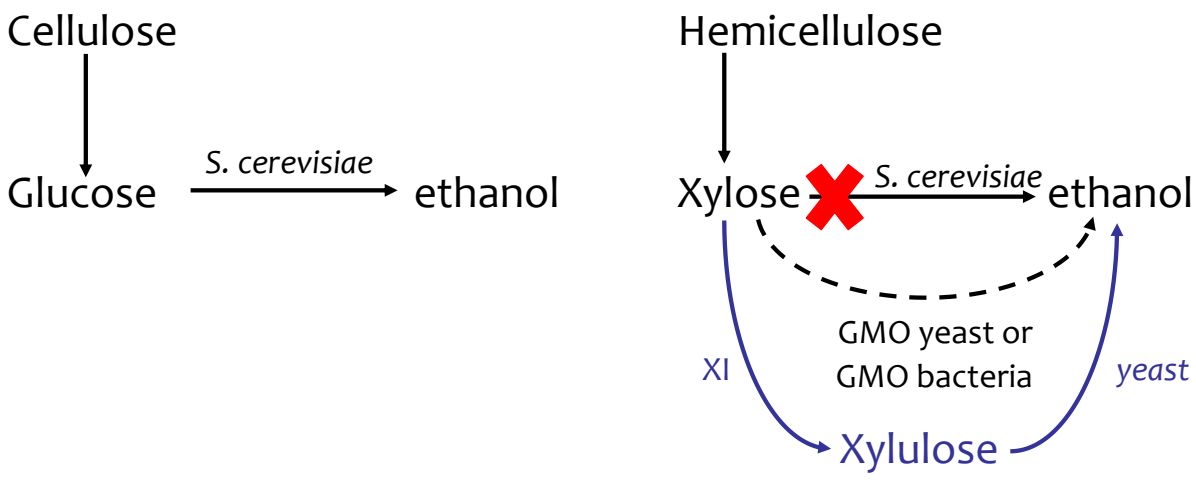


Figure 1. Our approach for fermentation of sugars to ethanol with native yeast.

to conferment glucose and xylose. We have taken an alternate approach in which xylose is isomerized to its ketoisomer xylulose exogenously and then advantage is taken of the fact that

native yeast can convert xylulose to ethanol. While the exogenous isomerization approach provides a better control over the reaction step, the issues that need to be resolved with its implementation are: 1) The unfavorable equilibrium associated with xylose/xylulose conversion (typically it is 5 parts of xylose to one part of xylulose), and 2) The need to recover and reuse the xylose isomerase (XI) enzyme.

In this project we developed two different strategies to deal with these issues (see Figure 2). The first strategy is simultaneous isomerization and fermentation, SIF, in the presence of soluble xyulose binding agents. This can be done on unfiltered or filtered hydrolysates following biomass pretreatment and saccharification. The advantages of using unfiltered hydrolysates are that it permits simultaneous saccharification and isomerization, in addition to SIF. Hence one can actually carry out SSF with native yeast. This approach, as will be described later, requires a specially designed biocatalyst (see Figure 3). However, in most cases, process economics mandate lignin recovery and hence the need to filter the hydrolysates, prior to fermentation. The specially designed biocatalyst pellets can be used to carry out SIF with filtered hydrolysates as well. Here, we can actually recover not only lignin but the catalysts pellets as well. Even yeast can be reused several times by using a non-traditional fermentor design. The second strategy that was developed with filtered hydrolysates is sequential isomerization and fermentation in presence of immobilized ketose-bind agents. This strategy does not require any specially-designed biocatalyst and, as such, commercially available immobilized XI pellets can be used. This approach allows recovery of the biocatalyst as well as the ketose-binding agents.

Strategy 1*: SIF in presence of soluble ketose binding agents

	Advantages	Special requirements
1a. Unfiltered hydrolysates	• In addition to SIF, also permits SSI • Hence, SSF becomes feasible	• Co-immobilized urease-and-XI pellets for isomerization at fermentation pH
1b. Filtered hydrolysates	• Permits SIF while allowing: • Recovery and reuse of the co-immobilized enzyme pellets • Recovery of lignin	• In addition to 1a, non-traditional fermentor configuration

Strategy 2**: Sequential I & F with immobilized ketose binding agents

Advantages	Special requirements
• Off-the-shelf XI/no co-immobilization with urease • Recovery and reuse of XI and ketose binding agent • Concentrate and purify xylulose	• pH adjustment between isomerization and fermentation

* S. Varanasi, K. Rao, P. Relue, and D. Yuan, "Methods of fermentation of xylose and hexose sugars," U.S. Pat. No. 8,507,232 B2, issued August 13, 2013; divisional U.S. Patent Application 13/955,270, filed July 31, 2013.
** Bin, Li, S. Varanasi, and Patricia Relue, "Aldose-Ketose Transformation for Separation and/or Chemical Conversion of C6 and C5 Sugars from Lignocellulosic Biomass Hydrolyzate, US Provisional Patent filed, Ser No:61/325,710, 04/2010

Figure 2. The two strategies implemented for fermentation of hydrolysates to ethanol with native yeast.

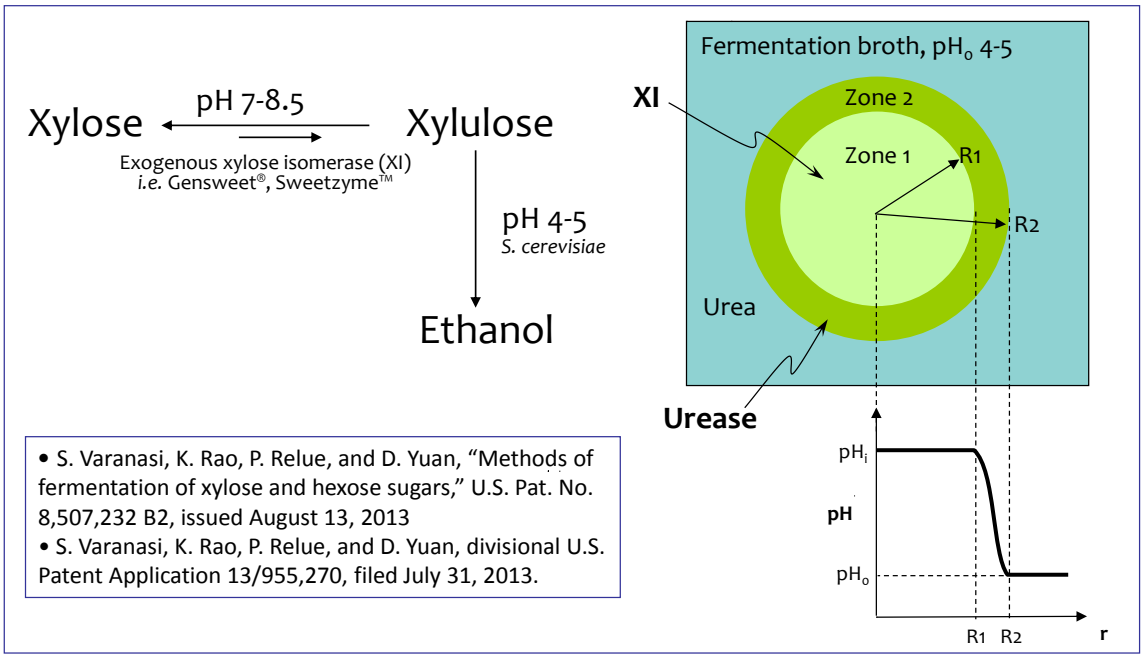


Figure 3: Method for sustaining two pH microenvironments in a single vessel.

For implementing these strategies, the following tasks with corresponding milestones were proposed.

- Task A: Co-immobilized enzyme pellet production
- Task B: Design of reactors
  - Packed bed immobilized enzyme pellet reactor (designed & performance optimized).
  - Fermentation modules (designed and tested for SIF).
- Task C: SSF with co-immobilized enzyme pellets and native yeast.
- Task D: New ketose binding agents
  - Identify and test their performance
  - Develop strategies for their recovery and reuse

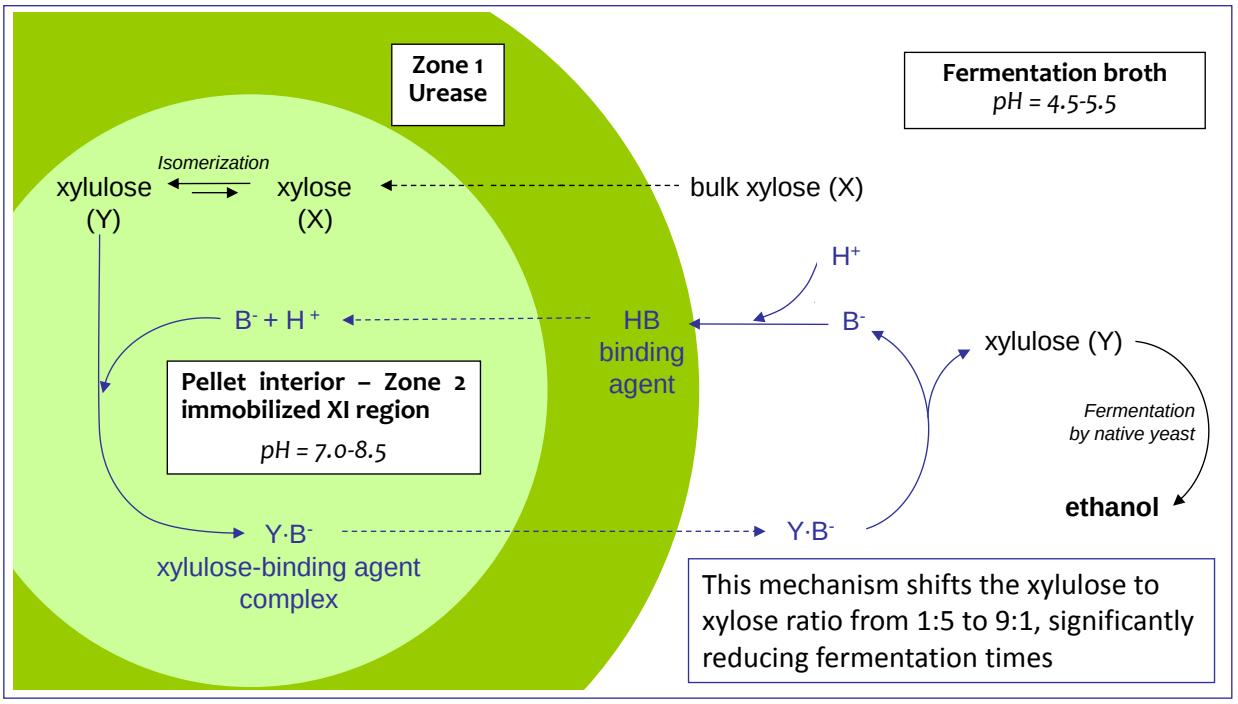
All the milestones associated with these tasks were successfully met during the project implementation. We next describe the technical accomplishments and results of the project.

- *Strategy 1: Simultaneous isomerization and fermentation (SIF)*

As previously noted, the isomerization reaction has an unfavorable equilibrium. Simultaneous isomerization and fermentation can drive the isomerization forward. However, pH of isomerization and fermentation are very different. We implemented a co-immobilized enzyme pellet strategy to deal with this situation. By coating commercially available XI pellets with a thin layer of urease and contacting these coated pellets with the fermentation medium in which urea is already present as a nitrogen source, we showed that it is possible to sustain different pH environments needed for SIF in a single vessel. Here, the ammonia produced by urea hydrolysis helps the maintenance of the pH gradient. Since fermentation is inherently slow and is the rate-

limiting step, SIF by itself does not drive the isomerization forward very effectively and the overall process remains slow. However, we can take advantage of this ability to generate pH-gradient to significantly enhance the isomerization by adding compounds capable of selectively binding to xylulose, namely boronic acids (see Figure 4; more details can be found in: Rao *et al*, *Applied Biochem Biotech*, 2008). The boronic acid, when added to the fermentation medium, diffuses into the pellet interior, where it ionizes in the prevailing neutral to alkaline pH environment. The conjugate-base complexes to xylulose and carries it from the pellet core to the broth. In the low pH environment in the broth, the complex dissociates releasing free xylulose to the broth. The free xylulose released to the broth provides high xylulose concentration for efficient ethanol production. The conjugate base is protonated to boronic acid in the low pH environment of the broth which diffuses back into the pellet core and the cycle is repeated. (See more details in Yuan *et al*, *Bioresource Tech*, 2011.)

While the bi-layered picture of the biocatalyst with an inner core of XI and an outer layer of urease helps to gain an appreciation of the physico-chemical phenomena behind the development of two-pH environment, a much simpler design where both XI and urease are co-immobilized on an imperious solid support as shown in Figure 5 will provide the needed two-pH environment. We used this simpler method of the two-enzyme pellet in our research. We identified and tested 3 commercial functionalized supports (Eupergit® 250c, Sepabeads® HA, and Sepabeads® EP) for XI and urease immobilization. We developed robust co-immobilized pellets by covalently attaching both the enzymes XI and urease to these supports via oxirane functional groups on



K. Rao; S. Chelikani; P. Relue; and. S. Varanasi, "A Novel technique for Optimizing the Simultaneous-Isomerization-and-Fermentation (SIF) Approach of Converting Xylose to Ethanol," *Applied Biochemistry and Biotechnology*, 146(1-3):101-117, 2008.

Figure 4: Enhancing conversion of xylose to xylulose in co-immobilized enzyme pellets via addition of a soluble ketose binding agent (HB).

their surface. We found good activity of both enzymes when immobilized on Sepabeads EP. The resulting biocatalyst particles did not show any loss of activity after repeated use with respect to the isomerization reaction or the urea hydrolysis reaction as shown in Figure 6. We used these robust biocatalysts in all our subsequent experiments.

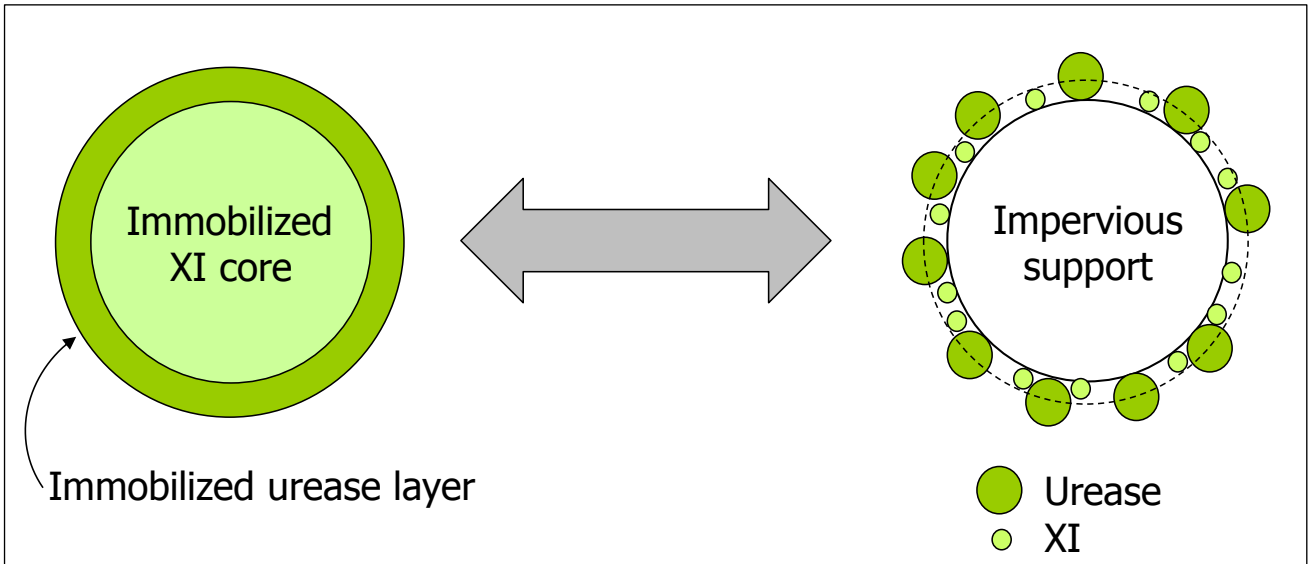


Figure 5: Schematic showing the enzyme localization in the bi-layered and co-immobilized enzyme pellets.

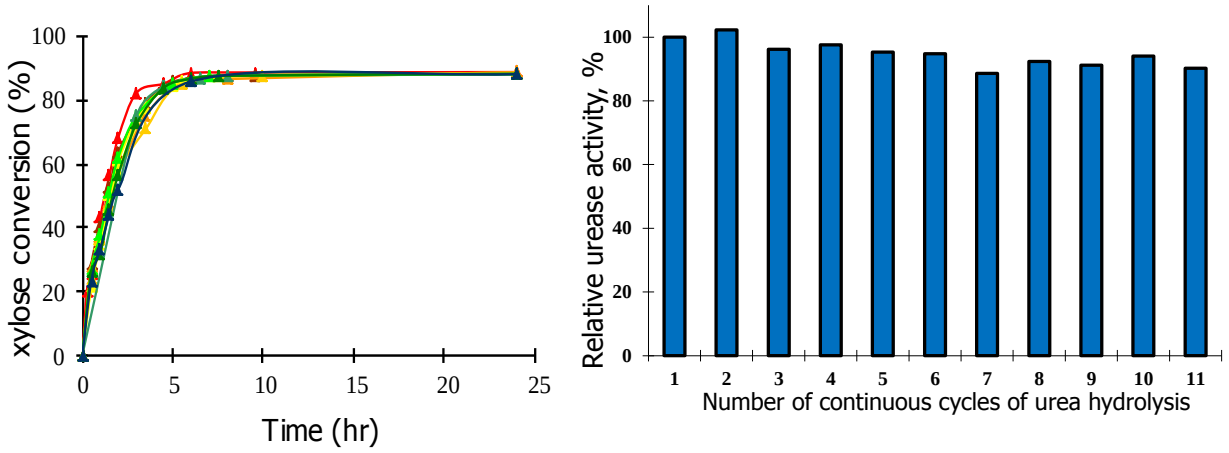


Figure 6: Biocatalyst development. The left panel shows Immobilized XI active after 14 days of continuous xylose isomerization, pH 7.5, where each line represents the reaction on a different day. The right panel shows immobilized urease activity for repeated cycles of urea hydrolysis, pH 7.5.

Strategy 1a: Unfiltered hydrolysate and soluble binding agent (SSF) (Milestone C)

Since the co-immobilized enzyme pellets are capable of sustaining two different pH environments and also are functional up to 60 °C, we first used them in a simultaneous saccharification and isomerization (SSI) application with biomass generated by two different pretreatment methods: dilute acid pretreatment and ionic liquid pretreatment. We show in Figure 7 results with dilute acid pretreated biomass. The left plot shows results with traditional saccharification while the right panel shows those for SSI. To achieve SSI, we added to the pretreated biomass the co-immobilized enzyme pellets, urea, and boronic acid in addition to the standard cellulase enzyme cocktail. As seen in Figure 7, both cellulose hydrolysis and isomerization of xylose take place concurrently in the medium containing co-immobilized enzyme pellets and cellulases.

Next, we demonstrated simultaneous saccharification and fermentation (SSF) by simply adding yeast to the saccharified biomass after lowering the temperature to 35°C with no pH adjustments. Figure 8 shows that both xylose isomerization and fermentation of glucose and xylulose do take place with native yeast. We successfully demonstrated that SSF is feasible with the co-immobilized enzyme pellets. An added benefit of combining saccharification with isomerization is that cellulase inhibition during saccharification may be alleviated

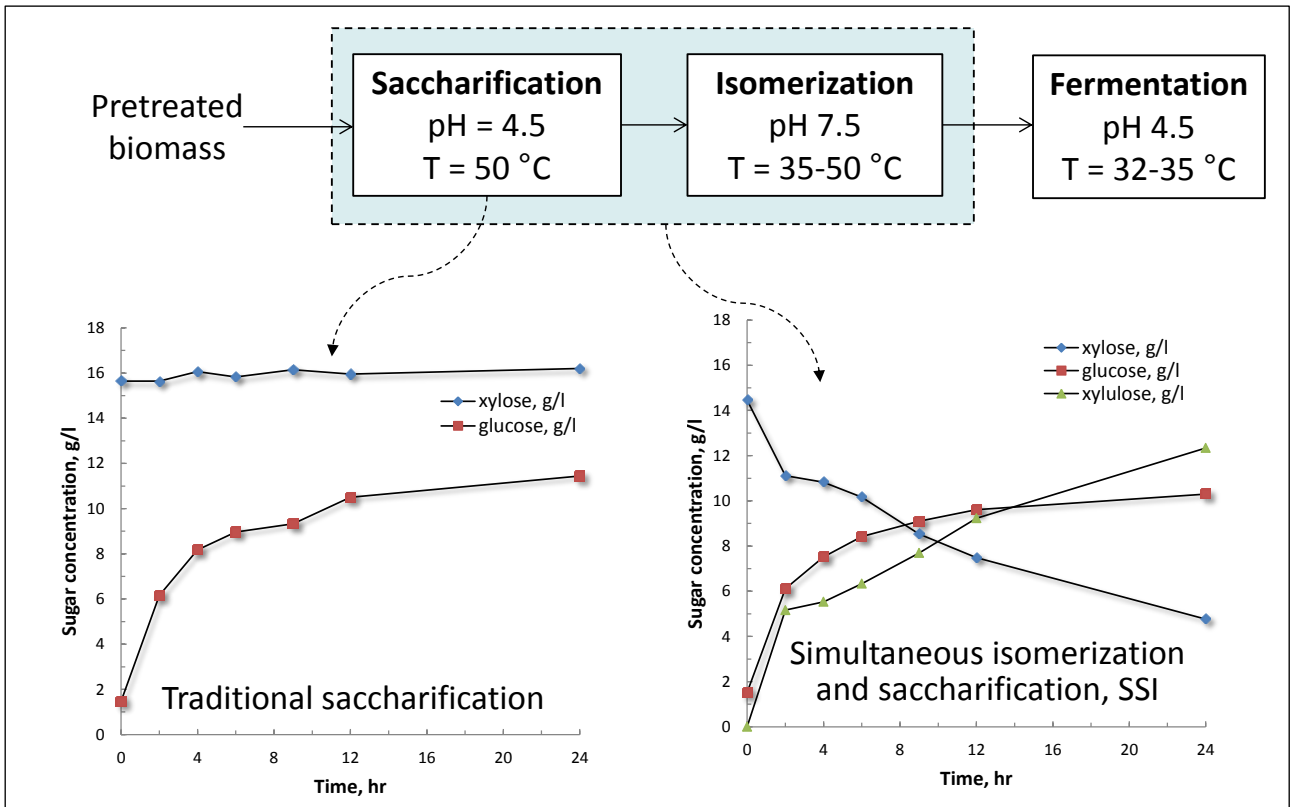


Figure 7: Traditional and SSI using unfiltered hydrolysate and soluble binding agent (SSI). After dilute acid pretreatment (1% H_2SO_4 with 7.5% solids loading at 120 °C for 60 min), the pH was adjusted to 4.5 with citrate buffer and the pretreated biomass was saccharified using a standard enzyme cocktail. For SSI, co-immobilized XI pellets, urea, and borax were also added.

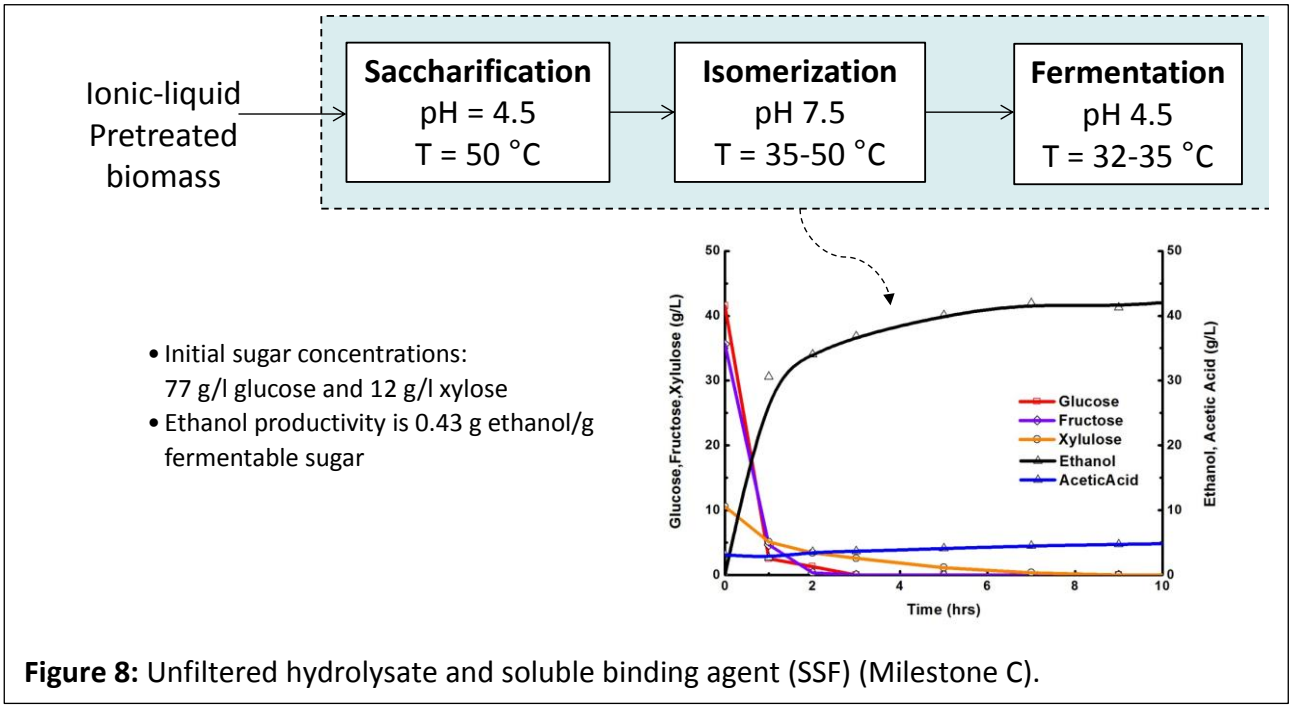


Figure 8: Unfiltered hydrolysate and soluble binding agent (SSF) (Milestone C).

In summary, for Strategy 1a, SSIF with native yeast was successfully demonstrated for producing ethanol from major lignocellulose sugars. In this approach, following separation of ethanol, residual is a complex mixture of yeast, lignin, borate, and saccharifying enzymes. Process economics warrants approaches that are capable of recovering and reusing most of these components as well as recovering lignin.

Strategy 1b: Filtered hydrolysate and soluble binding agent

Due to the process economics with respect to lignin recovery and use as a raw material as noted previously, we also implemented this strategy using filtered hydrolysates (1b). An overall process schematic is shown in Figure 9. In Strategy 1b, we filter the hydrolysates following saccharification to recover lignin, and then circulate the filtered hydrolysates repeatedly through

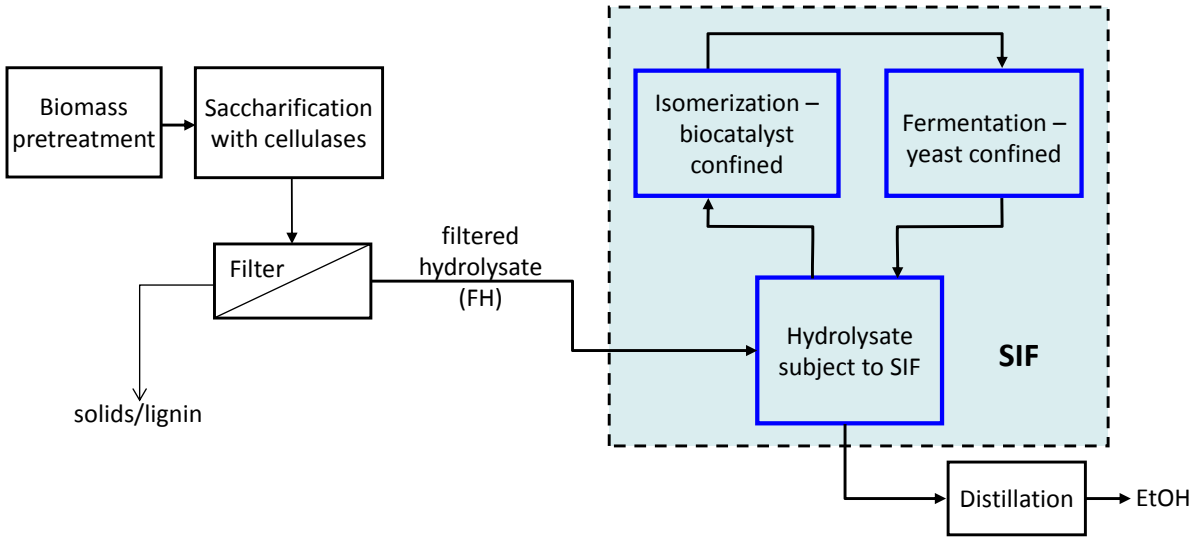
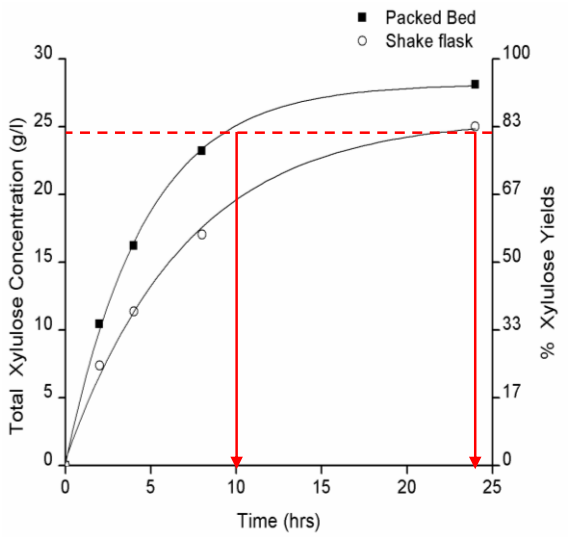


Figure 9: Process schematic for SIF with filtered hydrolysate and soluble binding agent.

two process units, (1) a packed bed of co-immobilized enzyme pellets, and (2) a fermentor designed to confine the yeast, to achieve SIF. This arrangement requires no pH adjustment between the two process units and permits recovery and reuse of XI pellets as well as the yeast.

We designed packed bed columns with co-immobilized pellets, and compared the performance of the column with isomerization experiments performed in shake-flasks using the same pellets. The system was designed for ease of recovery and reuse of the enzyme pellets during SIF. As can be seen in Figure 10, the kinetics of isomerization in the packed bed column are significantly faster than those observed in a shake flask due to improvements in mass transfer (10 hrs versus 24 hrs for 83% isomerization yield).

The fermentor design we chose to confine the yeast is a hollow fiber membrane fermentor (HFMF). Here, the yeast is confined to the shell-side and the sugar solution flows through the fiber lumen (see Figure 11, right panel). This design, which allows for packing yeast at high density in the fermentor, provides rapid fermentation and allows reuse of the yeast – we used the same yeast for up to 7 days with no reduction in ethanol yield. In HFMF, the by-product



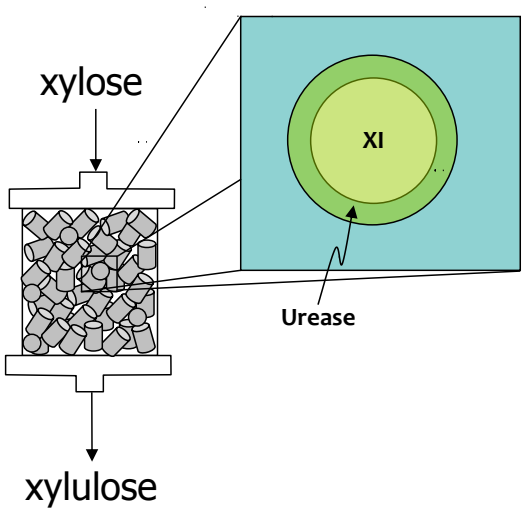
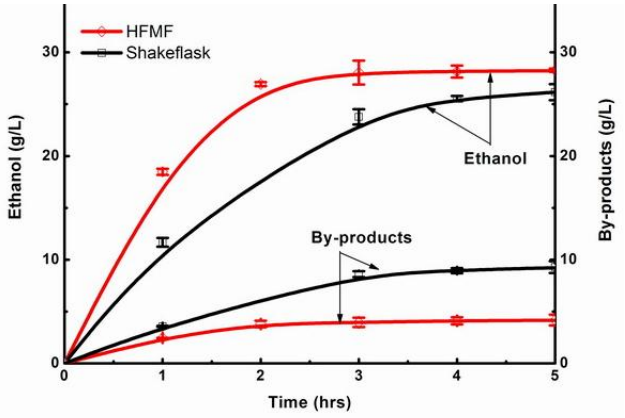


Figure 10: Improved isomerization kinetics in the packed bed enzyme column. Pellet loading was 18 g pellets/l of sugar solution, pH 4.5, 35 °C.



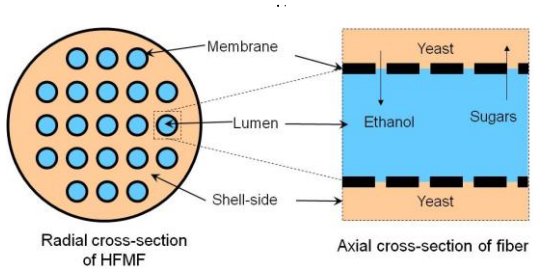


Figure 11: Hollow fiber membrane fermentor (HFMF) for confinement of yeast.

formation (glycerol and acetic acid) is also lower that that achieved in the shake-flask experiments as shown in the left panel of Figure 11.

As shown in Figure 12, our strategy with the filtered hydrolysates is the continuous circulation of the sugar solution through the packed column of biocatalyst particles and the HFMF to achieve SIF. As shown in Figure 13, we obtained an ethanol yield in the HFMF for biomass hydrolysates that was comparable to that achieved with model sugar mixtures. When the packed bed and HFMF were operated in other modes of circulation, as shown in Table 1 after 24 hours of fermentation, higher ethanol yields and concentrations were seen in SIF compared to the base case with no isomerization of xylulose. Also, if the hydrolysate was partially pre-isomerized prior to SIF, even higher sugar utilization and better ethanol yields were seen. (See more details in Yuan *et al*, *Bioresource Tech*, 2012.)

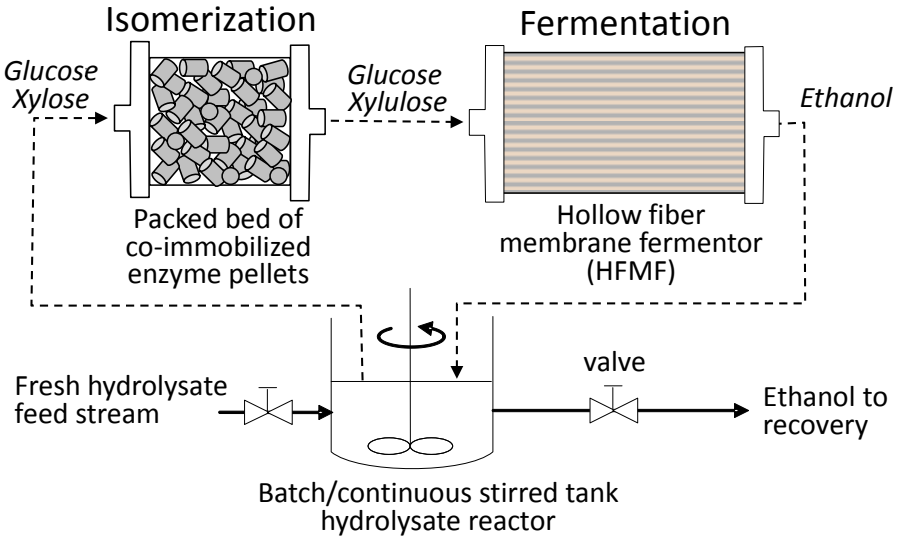


Figure 12: SIF of filtered hydrolysate (Milestone B).

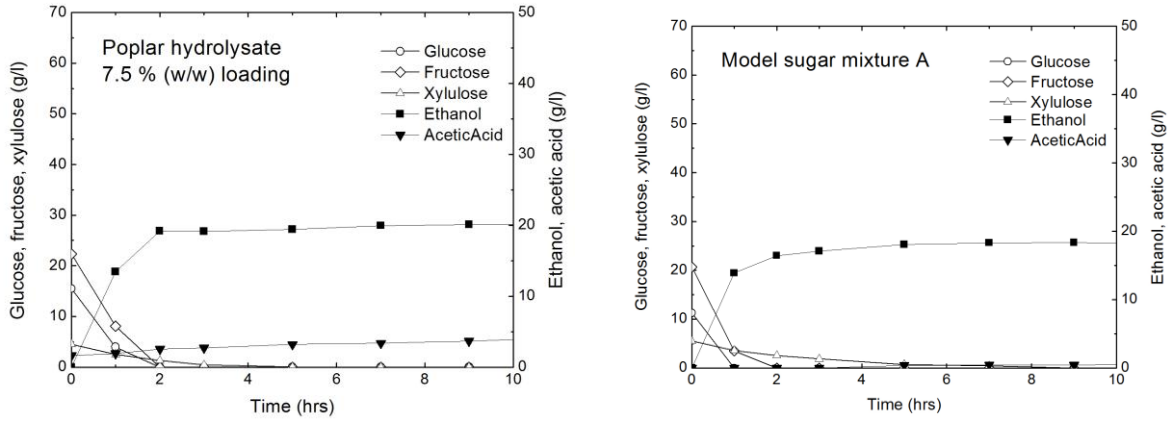


Figure 13: Fermentation of poplar hydrolysate and model sugar mixture using Hemoflow™ hollow fiber fermentor. Equivalent rates and extents of ethanol production were observed. Xylose not shown as at least 70% of xylose was isomerized to xylulose. The remaining xylose was converted to xylitol during fermentation.

Table 1: Results of SIF with filtered hydrolysates at 24 hrs.

Mode of Operation (all data is at 24 hrs)	Ethanol (g/l)	Ethanol yield (g ethanol/ g sugar)	Sugar utilization (60 g G+30 g X)	Xylitol (g/l)	Glycerol (g/l)	Acetic Acid (g/l)
No isomerization of xylose	30.93	0.34	84%	2.75	5.32	1.94
SIF	37.22	0.41	84%	2.07	5.32	1.95
Pre-isomerization of xylose (to 50%) followed by SIF	41.22	0.46	98%	2.61	4.92	1.94

- *Strategy 2: Sequential isomerization & fermentation (SIF) with immobilized ketose binding agent*

While Strategy 1b allows for recovery and reuse of the isomerization enzyme, the soluble ketose-binding agent remains in the fermentation medium. Economics and environmental concerns mandate recovery and reuse of the binding agent. We identified and tested several soluble binding agents that could selectively bind to xylulose. These include borate, germanate, phenylboronic acid (PBA), 3-amino PBA, and 4-carboxy PBA. These boronic acids are able to bind and unbind to xylulose, which is a polyol, through the pH-dependent esterification shown in Figure 14. This same mechanism is also responsible for the selective binding of xylulose by the binding agents borax and germanate.

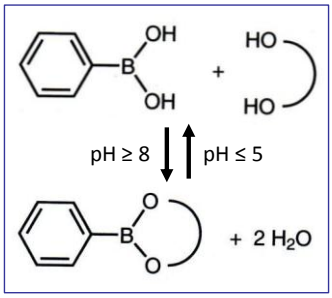


Figure 14: Reversible sugar binding of via pH-dependent esterification.

The results of xylose isomerization using XI in the presence of several xylulose binding agents are shown in Figure 15. Using binding agent concentrations with equivalent capacity for ester formation, xylulose conversion shows remarkable similarity for all compounds. Another aspect that must be accounted for in the evaluation of these agents is the ability of yeast to tolerate their presence. The biocompatibility of these agents was evaluated by studying their concentration-dependent effect on yeast growth rates when added to fermentation media. As shown in Table 2, germanate is the most biocompatible of the agents tested. All of the forms of PBA proved harmful

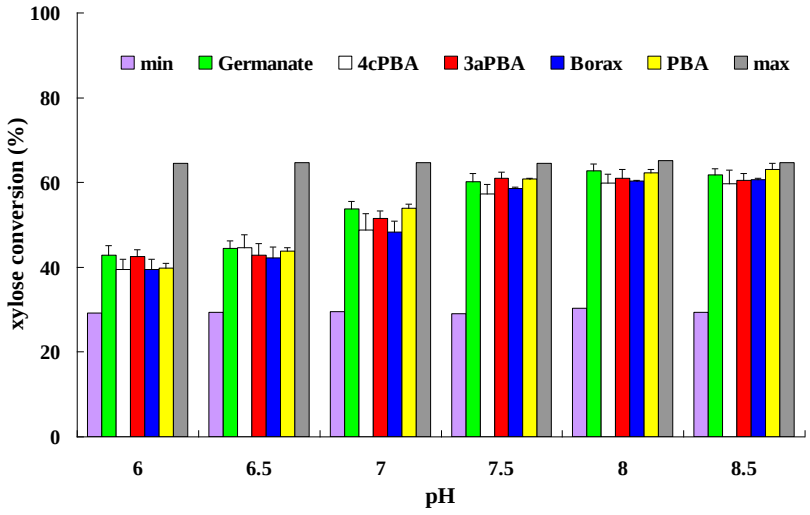


Figure 15: Comparison of performance of binding agents for xylulose binding efficacy. The concentrations were normalized to correspond to 0.05 M borax equivalents of binding sites. Maximum theoretical conversion (max) based on 100% complexation to xylulose.

Table 2: Evaluation of biocompatibility of xylulose binding agents under standard ethanol fermentation conditions with *S. cerevisiae*. RGR – relative growth rate of yeast (%) to control without any binding agent; OD is measured at 600 nm after 24hrs of culture. While germinate is the most biocompatible agent, all PBAs inhibit cell growth, and are toxic at higher concentrations.

[C]	Germanate		PBA		Borax	
	RGR	OD	RGR	OD	RGR	OD
0	100	3.72	100	3.72	100	3.72
5	98.06	3.73	77.88	2.28	85.92	
10	97.75	3.56	36.64	0.63	77.61	
20	97.01	3.61	15.86	0.28	60.61	
30	96.55	3.71	4.32	0.24	51.14	
40	96.97	3.66	0	0.26	45.58	

to yeast when in direct contact. Accordingly, we devised two methods for physically confining the binding agents to avoid their direct contact with the yeast. These methods, which are discussed next, provide the additional advantage of ease of recovery and reuse to improve process economics.

Although both aldoses and ketoses bind to boronic acids, ketoses generally bind with much higher affinity. The binding of two different boronic acids, PBA and N2B, to both aldose and ketose sugars is shown in Figure 16. At a pH of 8.5, which is compatible with isomerization of xylose to xylulose by XI, both show high selectivity for xylulose over all other sugars. To enable recovery and repeated reuse of these ketose binding agents, we implemented two different approaches.

In Strategy 2a, we confined the binding agent to an immiscible organic phase into which xylulose was selectively extracted while the isomerization occurred. This method we called simultaneous-isomerization-and-reactive-extraction (SIRE). Following SIRE, xylulose was back-extracted (BE) into a low pH medium as a concentrated stream for fermentation. Following back-extraction of sugars, the organic phase containing the binding agent can be reused. This strategy is referred to as SIRE-BE (details can be found in our recent publication: Li *et al*, *Green Chemistry*, 2012). In Strategy 2b, the recovery and reusability of the ketose binding agent is

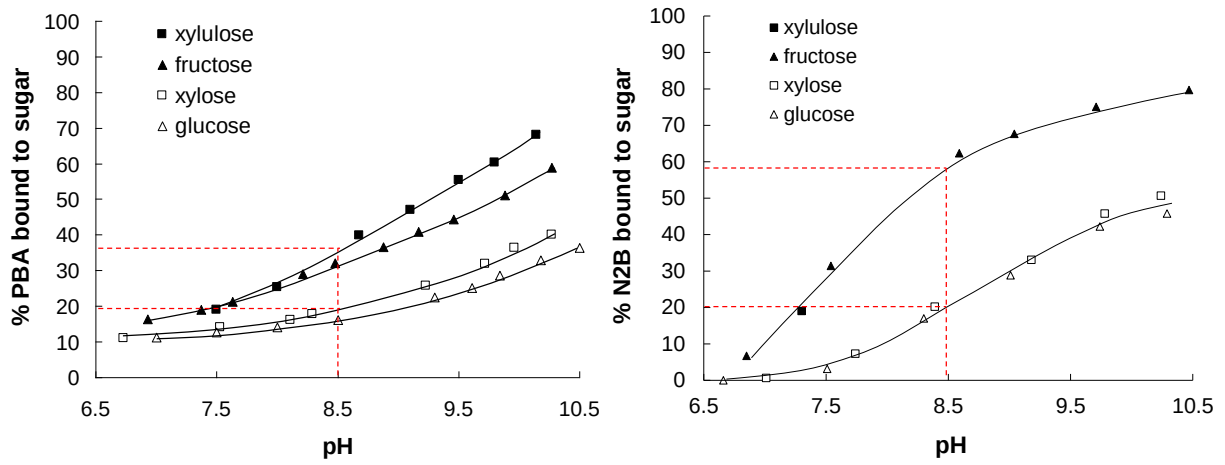


Figure 16: Relative binding of aldose and ketose sugars to PBA and N2B.

achieved through solid-phase extraction approach. Both approaches are described in more detail in the sections that follow.

Strategy 2a: Sequential isomerization & fermentation (SIF) with liquid-liquid, selective xylulose extraction

We implemented SIRE by contacting an aqueous reaction medium, in which xylose/xylulose transformation is catalyzed by suspended immobilized XI particles, with an organic phase comprised of naphthalene-2-boronic acid (N2B) and Aliquat® 336 dissolved in octanol (see Figure 17). Following SIRE, xylulose was back-extracted into a low pH HCl solution. Stripping was conducted with volume ratios of organic to stripping solution of 8:1. This combination allowed an 80% recovery of sugar into a five-fold concentrated stream that is a 90:10 mixture of xylulose:xylose. Through a staged stripping process developed based on the affinity differences of xylose and xylulose to N2B, a substantial improvement in the xylulose purity of the recovered stream was achieved (97% xylulose; see Table 3). By tailoring the organic phase composition, the extractability and selectivity of the sugars can be drastically improved. The sugars can be concentrated in both the extraction and stripping steps by reducing the volume ratio of the sugar-receiving phase relative to the sugar-donating phase for each step.

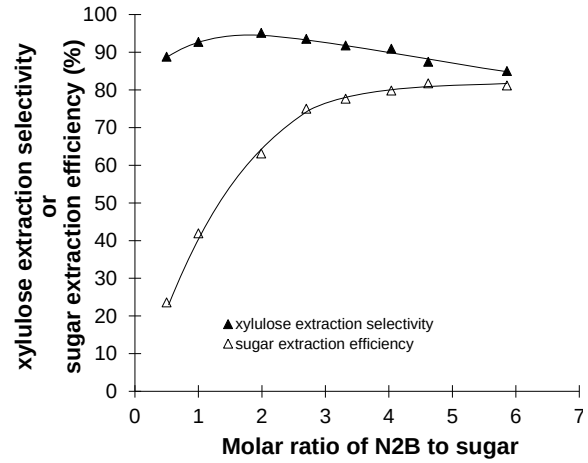


Figure 17. The effect of N2B to sugar molar ratio on SIRE with 10 mM xylose initially added to the aqueous phase. The organic phase used was pure 1-octanol with a fixed ratio of Aliquat® 336 to N2B of 2.5. Equal volumes of aqueous and organic phases were used.

Table 3. Table 1 Summary of SIRE-BE results for a very low concentration xylose stream. The net result of this process is the production of 5-fold concentrated xylulose solution (4) in acid media.

Phase	Volume (ml)	Xylose (g/l)	Xylulose (g/l)	Xylose (mg)	Xylulose (mg)
1. Initial sugar solution	100	**1.56**	0	156	0
2. Aqueous after SIRE	100	0.18	0.11	18	11
3. Stage 1 BE	12.5	0.71	0.38	8.9	4.8
4. Stage 2 BE	12.5	0.24	**7.47**	3	93
5. Organic after Stage 2 BE	100				17*

* Calculated based on mass balance closure.

SIRE with glucose works quite similar to that presented here with xylose. Differences in isomerization kinetics between glucose and xylose determine the composition of the product sugar stream when SIRE is implemented with mixed sugars. Based on our exploration in this area, the differences in isomerization kinetics in conjunction with the disparity between ketose/aldose binding to N2B indeed make separation and purification of individual ketose sugars possible. With regards to biomass hydrolyzate, this technology provides contaminant-free, concentrated ketose sugar streams without the need for energy-intensive separation and concentration schemes.

Strategy 2b: Sequential isomerization & fermentation (SIF) with solid-phase, selective xylulose extraction

An alternate configuration to liquid-liquid extraction to enable recovery and reuse of the sugar binding agent is, as noted before, solid-phase extraction. Here, boronic acids with pendent amine groups, such as 3-amino-PBA, are immobilized to oxirane-functionalized supports through the chemistry shown in Figure 18. The immobilized binding agent columns are extremely stable and retain their sugar binding capability indefinitely. In this mode, both the XI enzyme and the binding agent are confined in separate packed beds as shown in Figure 19. During the isomerization step, the saccharified biomass at a pH of about 8.5 is circulated through the two packed bed columns in series. In the first column xylose is isomerized to xylulose, which binds to the immobilized boronic acid. In the second step, the xylulose-laden binding agent column is washed with a low pH fermentation medium to release pure, concentrated xylulose into the medium. (See more details in Li *et al*, published US patent application, 2012.) For the sugar fermentation, native yeast is simply added to the medium.

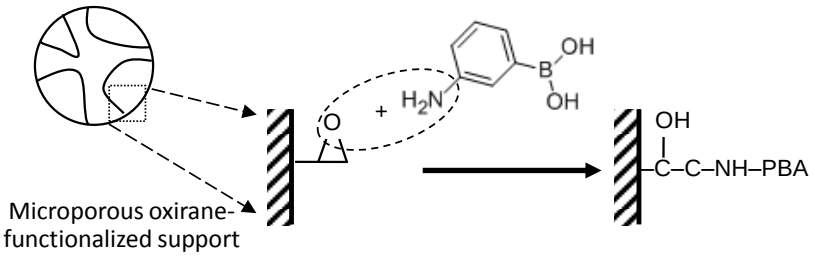


Figure 18: Immobilization of boronic acid to a solid support.

Results of xylulose fermentation to ethanol from the stripped xylulose sugar is shown in Figure 20. Clearly, fermenting xylulose by itself using native yeast is more efficient than co-fermenting xylulose with glucose. The fermentation times are reduced to less than half of that needed for co-utilization.

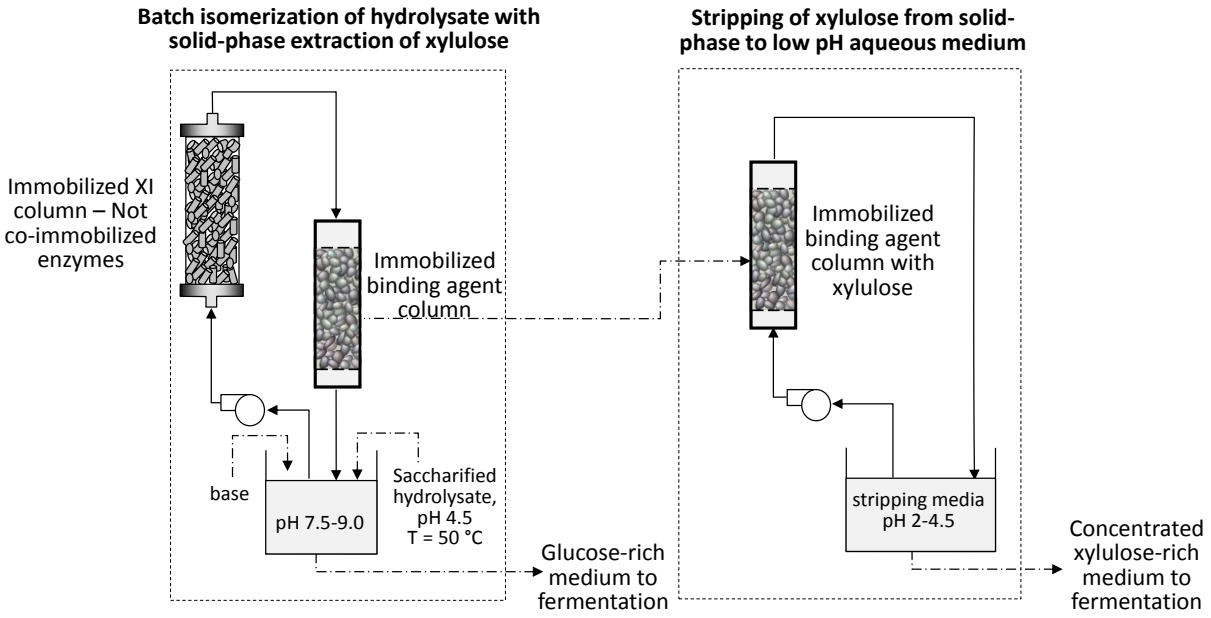


Figure 19: Two-step process for the isomerization and selective binding of ketose sugars and their release from immobilized boronic acid.

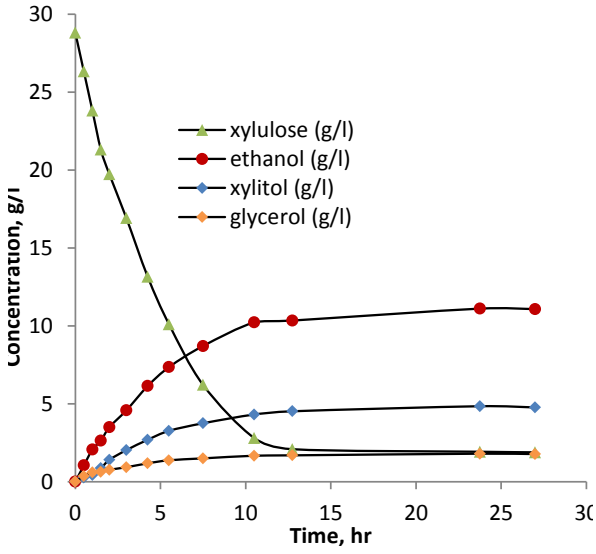


Figure 20: Results of xylulose fermentation using native yeast at pH 4.5 and 34 °C.

Strategy 2 has several advantages over using unfiltered hydrolysate. Since isomerization is sequential to fermentation, there is no longer a need for co-immobilized enzyme pellets. Fermentation can be conducted in a conventional fermentor; no special fermentor configurations such as HFMF are necessary. Separate fermentators for xylulose and glucose could be used to maximize yields and minimize ethanol fermentation times, thereby eliminating glucose-repression of C5 sugar uptake by *S. cerevisiae*. Strategy 2 is simpler to implement and is easily scalable while retaining the advantages of fermentation using non-GMO yeast. The confinement of the xylulose binding agent to an immiscible (solid or liquid) phase allows its repeated use as the immiscible phase can be contacted with fresh hydrolysates after sugar stripping. Finally, the ability to recover sugars in their concentrated, pure form allows their conversion to not only ethanol, but also other energy dense fuels such as furans (details can be found in our recent publication: Li *et al*, *Green Chemistry*, 2013).

6. Identify products developed under the award and technology transfer activities

- *Patents:*

S. Varanasi, K. Rao, P. Relue, and D. Yuan, "Methods of fermentation of xylose and hexose sugars," U.S. Patent Application 13/955,270, filed July 31, 2013.

B. Li, S. Varanasi and P. Relue. "Aldose-ketose transformation for separation and/or chemical conversion of C6 and C5 sugars from biomass materials". U.S. Patent Application 20130074397, PCT/US11/33030, November 12, 2012.

S. Varanasi, K. Rao, P. Relue, and D. Yuan, "Methods of fermentation of xylose and hexose sugars," U.S. Pat. No. 8,507,232 B2, issued August 13, 2013.

- *Refereed Journal Publications:*

B. Li, S. Varanasi, and P. Relue, "New route for enabling utilization of C5 and C6 sugars of lignocellulosic biomass by native *S. cerevisiae* for ethanol production," *in preparation for Bioresource Technology.*

B. Li, S. Varanasi, and P. Relue, "High yield aldose-ketose transformation for isolation and facile conversion of biomass sugar to furan," *Green Chemistry*, 15 (8): 2149 – 2157, 2013.

B. Li, P. Relue, and S. Varanasi, "Simultaneous isomerization and reactive extraction of biomass sugars for high yield production of ketose sugars," *Green Chemistry,* 14:2436-2444, 2012.

D. Yuan, K. Rao, S. Varanasi, and P. Relue, "A viable method and configuration for fermenting biomass sugars to ethanol using native *Saccharomyces cerevisiae*," *Bioresource Technology*, 117:92-98, 2012.

D. Yuan, K. Rao, P. Relue, and S. Varanasi, "Fermentation of biomass sugars to ethanol using native industrial yeast strains," *Bioresource Technology,* 102(3):3246–3253, 2011.

K. Rao, S. Chelikani, P. Relue, and. S. Varanasi, "A Novel technique for Optimizing the Simultaneous-Isomerization-and-Fermentation (SIF) Approach of Converting Xylose to Ethanol," *Applied Biochemistry and Biotechnology*, 146(1-3):101-117, 2008.

- *Conference presentations and posters: (FIRST AUTHOR IS PRESENTER)*

B. Li, K. Marbaugh, P. Relue and S. Varanasi, "Xylulose to Furfural, a New Route to Produce High Yield of Furan," AICHE Annual Meeting, paper 559b, November 3-8, 2013, San Francisco, CA.

H. Shao, S. Varanasi, P. Relue, and S. Viamajala, "Detoxification of Dilute-Acid and Ionic-Liquid Pretreated Wheat Straw Hydrolysates to Improve Succinic Acid Fermentation," AICHE Annual Meeting, paper 220a, November 3-8, 2013, San Francisco, CA.

K. Marbaugh, B. Li, S. Varanasi and P. Relue, "Mathematical modeling of furfural production from high purity xylulose," 35th Symposium of Biotechnology for Fuels and Chemicals, Poster 18-23, April 29-May 2, 2013, Portland, OR.

B. Li, P. Zhang, S. Varanasi and P. Relue, "Furfural production from xylulose in a bi-phasic reaction system," 35th Symposium of Biotechnology for Fuels and Chemicals, Poster 18-24, April 29-May 2, 2013, Portland, OR.

P. Zhang, B. Li, H. Shao, S. Varanasi and P. Relue, "2,3-Butanediol production from xylulose fermentation," 35th Symposium of Biotechnology for Fuels and Chemicals, Poster 2-14, April 29-May 2, 2013, Portland, OR.

B. Li, P. Zhang, S. Varanasi and P. Relue, "Simultaneous isomerization and reactive extraction of biomass sugars for efficient furan production via high yield ketose intermediate," 35th Symposium of Biotechnology for Fuels and Chemicals, Poster 3-45, April 29-May 2, 2013, Portland, OR.

B. Li, S. Varanasi, and P. Relue, "High yield ethanol production from fermentation of C5 and C6 biomass sugars by using native yeast," AICHE Annual Meeting, paper 211b, October 2012, Pittsburgh, PA.

K. Marbaugh, S. Varanasi, and P. Relue, "Separation of sugars from biomass hydrolysates using plasticized liquid membranes," AICHE Annual Meeting, paper 505c, October 2012, Pittsburgh, PA.

P. Relue, B. Li, and S. Varanasi, "One-pot homogeneous synthesis of furfural from high purity xylulose in aqueous media," AICHE Annual Meeting, paper 129c, October 2012, Pittsburgh, PA.

H. Shao, B. Li, P. Relue, and S. Varanasi, "Detoxification of ionic-liquid pretreated wheat straw hydrolysate improves succinic acid fermentation performance," 34th Symposium of Biotechnology for Fuels and Chemicals, Poster 13-28, April 30-May 3, 2012, New Orleans, LA.

K. Marbaugh, S. Varanasi, and P. Relue, "Characterization of plasticized liquid membranes with application for sugar separation," 34th Symposium of Biotechnology for Fuels and Chemicals, Poster 13-30, April 30-May 3, 2012, New Orleans, LA.

B. Li, P. Relue, and S. Varanasi, "Simultaneous Isomerization and Reactive Extraction (SIRE) of Xylose for High Yield Production of Xylulose," 34th Symposium of Biotechnology for Fuels and Chemicals, Poster 13-33, April 30-May 3, 2012, New Orleans, LA.

B. Li, P. Relue, S. Varanasi, "Reactive extraction of biomass sugars for more efficient downstream conversion to products, AIChE Annual Meeting, Minneapolis, MN, October 2011.

H. Shao, B. Li, P. Relue, S. Viamajala, and S. Varanasi, "Efficient fermentation of biomass sugars to succinic acid," 33rd Symposium on Biotechnology for Fuels & Chemicals, Seattle, WA, May 2011.

P. Relue, B. Li, D. Yuan, H. Shao, and S. Varanasi. "Evaluation of Oxianions for Shifting the Xylose:Xylulose Equilibrium with Immobilized Xylose Isomerase," AIChE Annual Meeting, Salt Lake City, UT, November 2010.

P. Relue, B. Li, D. Yuan, H. Shao, and S. Varanasi. "Evaluation of Immobilized Boronic Acid for Shifting the Xylose:Xylulose Equilibrium with Immobilized Xylose Isomerase," AIChE Annual Meeting, Salt Lake City, UT, November 2010.

D. Yuan, B. Li, H. Shao, S. Varanasi and P. Relue. "Adaptation of Native *S. cerevisiae* for Improved Xylulose Utilization", 32nd Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, FL, April 2010.

B. Li, D. Yuan, H. Shao, P. Relue and S. Varanasi. "Immobilization of urease for a microenvironmental pH control system: effect of media additives on enzyme activity". 32nd Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, FL, April 2010.

H. Shao, D. Yuan, B. Li, P. Relue and S. Varanasi. "Efficient fermentation of biomass sugars to lactic acid". 32nd Symposium on Biotechnology for Fuels and Chemicals, Clearwater Beach, FL, April 2010.

B. Li, D. Yuan, P. Relue and S. Varanasi. "Theory for dual-layer co-immoblization of Xylose Isomerase with Urease-coated". UT Midwest Graduate Research Symposium, Toledo, OH, March 2010.

D. Yuan, B. Li, S. Varanasi and P. Relue. "Efficient Bioconversion of Glucose and Xylose From Popar Hydrolysate to Ethanol," UT Midwest Graduate Research Symposium, Toledo, OH, March 2010.

D. Yuan, B. Li, S. Varanasi and P. Relue. "A Native S. cerevisiae Fermentation Methodology for Converting Glucose and Xylose From Biomass Hydrolysate to Ethanol", AIChE Annual Meeting, Nashville, TN, November 2009.

B. Li, D. Yuan, P. Relue and S. Varanasi. "Evaluation of Enzyme Immobilization Methods for Xylose-to-Xylulose Isomerization". AIChE Annual Meeting, Nashville, TN, November 2009.

D. Yuan, P. Relue, and S. Varanasi, "A Viable Method for Fermenting Both Glucose and Xylose to Ethanol Using Native Yeast," 31st Symposium on Biotechnology for Fuels & Chemicals, San Francisco, CA, May 2009.

P. Relue and S. Varanasi, "A novel SIF strategy for co-fermentation of C5 and C6 sugars with native yeast," DOE Office of the Biomass Program, Biochemical Platform Review Meeting, Denver, CO, April 2009.

D. Yuan, P. Relue, and S. Varanasi, "A Simultaneous Isomerization and Fermentation (SIF) Process for Co-fermentation of Glucose and Xylose," UT Graduate Student Association Research Symposium, Toledo, OH, *received 1st place poster award*, April 2009.

B. Li, D. Yuan, S. Varanasi, and P. Relue, "Xylose isomerization to xylulose with urease-coated xylose isomerase," UT Graduate Student Association Research Symposium, Toledo, OH, *received honorable mention poster award*, April 2009.

K. Rao, S. Varanasi, P. Relue, "A Novel Approach for Conversion of Xylose to Ethanol Using Native Strains of Yeast," 30th Symposium on Biotechnology for Fuels & Chemicals, New Orleans, LA, May 2008.

- *Invited Presentations:*

"Sustainability, energy and options: It's all a matter of time," P. Relue, Toledo Early College High School Career Symposium, April 20, 2012.

"Sustainability and Energy: Making Transportation Fuels from Biomass," S. Varanasi, Issue Day Community Workshop, Maumee Valley Country Day High School, March 8, 2012.

"Sustainability: A Panel Discussion," M. Franchetti, G.G. Lipscomb, and P. Relue, UT Theta Tau Engineering Fraternity, March 21, 2011.

"Biomass to Fuel," UT ACS Chemistry Club, P. Relue, Toledo, OH, April 26, 2010.

"Forest Biomass to Fuel," P. Relue and S. Varanasi, Society of American Foresters (Ohio Chapter), Spring Annual Meeting, OSU Campus, Columbus, OH, March 18, 2009.

- *PhD Dissertations*

Mr. Bin Li, "Innovative Methods for Improving Biomass Sugars Utilization," Sept. 2012 (P. Relue and S. Varanasi, co-advisors).

Mr. Dawei Yuan, "Strategies for Efficient Fermentation of Biomass Derived Glucose and Xylose to Ethanol using Naturally Occurring Saccharomyces cerevisiae," Nov. 2010 (P. Relue and S. Varanasi, co-advisors).