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A Novel Technique that Enables Efficient Conduct of Simultaneous Isomerization and Fermentation (SIF) of Xylose

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Abstract Of the sugars recovered from lignocellulose, D-glucose can be readily converted into ethanol by baker's or brewer's yeast (*Saccharomyces cerevisiae*). However, xylose that is obtained by the hydrolysis of the hemicellulosic portion is not fermentable by the same species of yeasts. Xylose fermentation by native yeasts can be achieved via isomerization of xylose to its ketose isomer, xylulose. Isomerization with exogenous xylose isomerase (XI) occurs optimally at a pH of 7–8, whereas subsequent fermentation of xylulose to ethanol occurs at a pH of 4–5. We present a novel scheme for efficient isomerization of xylose to xylulose at conditions suitable for the fermentation by using an immobilized enzyme system capable of sustaining two different pH microenvironments in a single vessel. The proof-of-concept of the two-enzyme pellet is presented, showing conversion of xylose to xylulose even when the immobilized enzyme pellets are suspended in a bulk solution whose pH is sub-optimal for XI activity. The co-immobilized enzyme pellets may prove extremely valuable in effectively conducting "simultaneous isomerization and fermentation" (SIF) of xylose. To help further shift the equilibrium in favor of xylulose formation, sodium tetraborate (borax) was added to the isomerization solution. Binding of tetrahydroxyborate ions to xylulose effectively reduces the concentration of xylulose and leads to increased xylose isomerization. The formation of tetrahydroxyborate ions and the enhancement in xylulose production resulting from the complexation was studied at two different bulk pH values. The addition of 0.05 M borax to the isomerization solution containing our co-immobilized enzyme pellets resulted in xylose to xylulose conversion as high as 86% under pH conditions that are suboptimal for XI activity. These initial findings, which can be optimized for industrial conditions, have significant potential for increasing the yield of ethanol from xylose in an SIF approach.

Keywords Xylose · Xylulose · Urea · Urease · Borate · Simultaneous isomerization and fermentation · Ethanol

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Introduction

Ethanol is being hailed as the fuel of the future. Interest in the production of fuel ethanol from renewable sources has increased significantly. For fuel ethanol production to become a practical reality, cheaper substrates and more efficient production processes are needed [1, 2]. Biomass, which includes all plant and plant-derived material, forms a potential renewable source of sugars that can be fermented to produce fuel ethanol and a variety of other fuels and chemicals. In addition to the many benefits common to renewable energy, biomass is particularly attractive because it is currently the only renewable sustainable energy source for liquid transportation fuel.

Lignocellulosic biomass consists of three major components: cellulose (~40–50%), hemicellulose (~25–35%), and lignin (~15–20%) [3]. Of these, cellulose and hemicellulose constitute the polysaccharides that can be hydrolyzed to sugars that could be fermented to ethanol. In biomass, the majority of cellulose is a crystalline polymer of glucose that is relatively difficult to hydrolyze into its monomeric sugar residues. Hemicellulose is a short-branched polymer of pentose and some hexose sugars that surround the cellulose fibrils and is much less organized [4]. The pentose sugars consist primarily of xylose and to a smaller extent arabinose, whereas the hexose sugars are usually galactose and mannose. Because of its relatively open structure, the hemicellulose fraction is easier to convert to its sugar monomers by various pretreatment techniques than the cellulose fraction.

For the conversion of lignocellulosic biomass to bioethanol to be economically feasible, it is imperative that the hemicellulose-derived monomeric sugars be fermentable along with the glucose derived from cellulose. Unfortunately, no known native microorganisms are able to efficiently ferment both glucose and xylose to ethanol. Wild-type *Saccharomyces cerevisiae* strains can readily ferment glucose and other sugar components of biomass like mannose, fructose, and galactose [5]. Xylose, which forms a major portion of hemicellulose, cannot be fermented by the same native strains of yeast. Several non-*Saccharomyces* strains of yeast, such as *Pichia stipitis* and *Candida shehatae*, are known to ferment pentose sugars more efficiently than other yeasts [6]. In such yeasts, the xylose metabolism pathway goes from xylose to xylitol to xylulose [7, 8]. In other yeast strains and bacteria and fungi, xylose can also be converted to xylulose via a single enzyme, xylose isomerase (XI). Several yeasts, including *S. cerevisiae*, that cannot ferment xylose are able to ferment xylulose, the ketose isomer of xylose [9–12]. Considerable effort has been focused on the genetic modification of microorganisms so that both xylose and glucose can be efficiently metabolized using the same organism [13–25].

Although genetically modified organisms have potential for fermentation of pentose and hexose sugars, their genetic stability, overall ethanol yield, and ability to survive under the conditions of industrial fermentation are unproven [26, 27]. Hence, an alternative approach to fermentation of xylose to ethanol involves using native yeast strains with the addition of exogenous enzymes for the isomerization of xylose. In this approach, the production of xylulose is accomplished using immobilized glucose/xylose isomerase [11, 28–30]. The appeal for this approach is that XI, along with amylase and protease, is among the most widely and cheaply available commercial enzyme [31]. Hydrolysate from lignocellulosic biomass will contain both xylose and glucose. The affinity of XI for xylose is typically 1 to 2 orders of magnitude greater than its affinity for glucose; hence, isomerization of xylose to xylulose will dominate over isomerization of glucose to fructose [31]. However, any fructose formed is readily fermentable by *Saccharomyces* to produce ethanol, so fructose formation is not a cause for concern.

Although XI is capable of converting xylose to xylulose, under conditions where XI has significant activity, the equilibrium ratio of xylose/xylulose is typically high (on the order

of 5:1) [32–34]. Hence, xylose isomerization does not have a favorable forward equilibrium. One way to increase xylose conversion is to drive the isomerization forward by removal of the product xylulose. Simultaneous isomerization and fermentation (SIF), where the isomerization of xylose and the fermentation of xylulose to ethanol occur simultaneously in the same vessel, is one method for increasing xylose utilization. However, SIF does have inherent limitations because of the pH range over which XI is active. All commercially available XI's have optimal activity at pH 7 to 8, and the XI activity drops sharply as the pH decreases. In contrast, the optimal pH for the fermentation is in the range of 4 to 5. The large pH difference associated with these two steps poses a problem for conducting SIF efficiently. The SIF can be carried out at a compromised pH between 4 and 7, but the results are less than optimal for both reactions [11]. Efforts to isolate a XI with optimal activity at significantly lower pH for SIF were also noted in the literature [30]. However, it does not appear that this enzyme has the same level of activity as displayed by the commercially available enzymes.

To overcome the disparity in the optimal pH's for the isomerization and fermentation, our group [29, 35, 36] proposed a novel scheme of isomerization that incorporates urease co-immobilized with xylose isomerase. This technique uses XI immobilized in a porous pellet for isomerization and the immobilized urease enzyme for pH control (Fig. 1). These co-immobilized enzyme pellets are dispersed in a fermentation broth, which contains urea in addition to the other necessary ingredients for fermentation. Theoretically, it is possible to sustain a significant pH gradient between the bulk liquid and the core region of the pellet

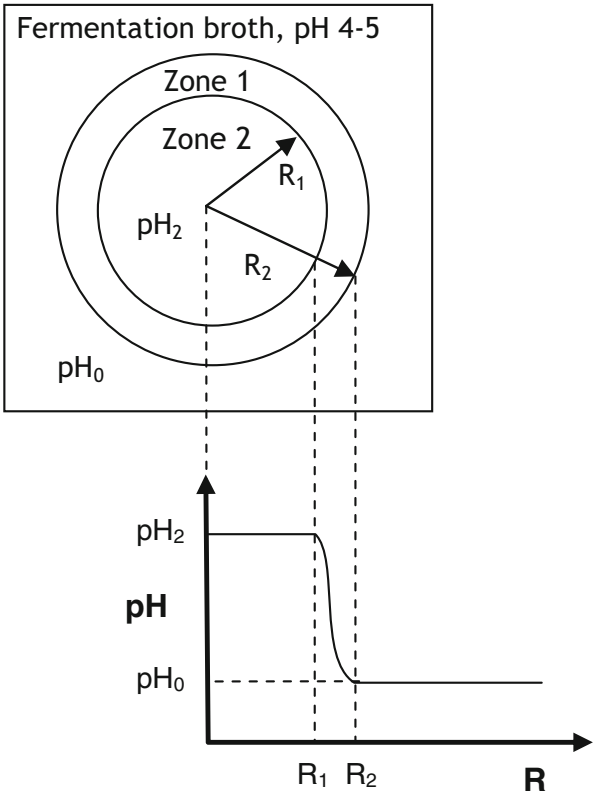


Fig. 1 Cross-section of a Sweetzyme™ pellet showing the steady-state pH profile developed when urease is co-immobilized in the pellet and urea is added to the fermentation broth. The pH in the fermentation broth is pH_0, which is typically in the range of 4 to 5. Zone 1 (outer layer) of the pellet contains immobilized urease and represents the region of the pellet where the pH changes with radial position as ammonia is produced by the consumption of urea. Zone 2 (core) represents the region of the pellet which is at pH_2, the elevated pH. The boundary between zones 1 and 2 represents either the point where all urea is consumed or the penetration depth of urease into the pellet

[29] because as hydrogen ions diffuse into the pellet, they are neutralized by the ammonia produced in the hydrolysis of urea by urease. The XI, which is maintained at a higher pH in the inner core of the pellet, then catalyzes the isomerization of the xylose to xylulose; xylulose diffuses from the pellet and is then available for fermentation in the bulk solution.

Although our co-immobilized enzyme approach is able to sustain the necessary pH difference between isomerization and fermentation steps in SIF [35], the overall production rate of ethanol in SIF will still be limited by the total concentration of xylulose available to the yeast [9]. Under normal equilibrium conditions, the xylulose concentration is usually at best one fifth of the xylose concentration. Hence, other avenues of shifting the equilibrium towards higher xylulose formation will further increase the rate of ethanol production.

One effective means of shifting the equilibrium toward increased xylulose formation is the addition of sodium tetraborate to the isomerization media [37–39]. Borate ion has been reported to shift the equilibrium between xylose and xylulose in XI catalyzed isomerization from 80:20 to 30:70. The borate ion binds more tightly to xylulose than xylose, effectively reducing the product concentration, and thus shifts the equilibrium toward increased xylulose formation. Interestingly, this ability of borate to bind to xylulose is pH dependent, with higher pH (6 to 7.5) favoring binding [40]. Thus, as the pH increases, the concentration of free xylulose decreases. Therefore, the rate of fermentation of xylulose in the presence of borate is also pH dependent, with lower pH leading to higher free xylulose concentrations and thus higher yields and rates of ethanol production [40]. In this regard, our co-immobilized enzyme strategy, which provides different microenvironments for isomerization and fermentation, is naturally benefited by the addition of borate to the fermentation broth. Inside the pellet, the pH is elevated, XI is active, and the isomerization equilibrium is favored by strong borate binding to xylulose. In contrast, in the low pH fermentation broth, borate has a reduced binding to xylulose and thus produces a higher free xylulose concentration for fermentation to ethanol.

In previous papers and patents, we have introduced our novel system and provided proof of concept for the co-immobilized enzyme pellet system [29, 35, 36, 49, 50]. In this paper, we present results of experiments that illustrate the effectiveness of our co-immobilized enzyme system for isomerization under conditions optimal for fermentation by common *S. cerevisiae*. We have changed the media composition in an effort to both shift the equilibrium in favor of xylulose production and improve XI activity [51]. In addition to borate, we also investigated the effect of metal ion addition to the kinetics and equilibrium of the isomerization reactions as certain divalent metal ions have been shown to increase the long-term activity of XI [41, 42]. This paper provides results of these experiments in our co-immobilized enzyme system and their potential impact on SIF enhancement.

Materials and Methods

Chemicals Novo Sweetzyme™ (Sigma Aldrich G4166 ≥350 U/g with activity based on isomerization of glucose to fructose), which is immobilized glucose isomerase produced from *Streptomyces murinus*, was used for the isomerization of xylose. The glucose isomerase has optimal activity for glucose/fructose isomerization at pH 7.5 and 60°C (as per the manufacturer). The Sweetzyme™ pellets were dry, brown, cylinder-shaped granules with a diameter of approximately 1–3 mm. Jack bean urease (Sigma U4002, 70,400 U/g) was used for generating the co-immobilized enzyme pellets used in the isomerization studies. Urease has optimal activity at pH 7.0 and 25°C (as per manufacturer). Both enzymes were stored at 4 °C.

Additional chemicals, including xylose, urea, borax, magnesium chloride, cobalt chloride, sodium citrate, and Tris were all purchased from Sigma Aldrich (St. Louis, MO).

Immobilization of Urease on Sweetzyme™ Pellets For co-immobilization of urease on the Sweetzyme™ pellets, 500 ml of 1 g/l urease solution and 2 g of Sweetzyme pellets was added to a 1-l beaker [35]. The beaker was left on the benchtop at room temperature for 24 h. The pellets were separated from the solution by decanting and gravity filtration and dried on a paper towel at room temperature for 24 h or until dry. Co-immobilized pellets were stored at 4 °C until use. Activity of immobilized urease was measured at pH 7.5 and 25°C using a standard assay procedure that measures the rate of ammonia liberation [43]. The urease activities obtained with our immobilization procedure were in the range of 550–577 U/g pellets, where a unit liberates 1 μmol of ammonia per minute under the assay conditions.

Measurement of Xylose Isomerization Kinetics and Equilibrium All experiments were carried out at 34 °C in a volume of 25 ml in 50-ml shake flasks agitated at 130 rpm in an incubated shaker. Each experiment was conducted in duplicate. All experiments used 60 g/l xylose, and unless otherwise noted, 5.2 g/l of enzyme pellets (0.13 g) was used for each experiment. Buffered solutions used in making the isomerization media were 0.01 M Tris buffer (pHed to 7.5 using 0.01 M NaOH) and 0.05 M sodium citrate buffer (pHed to 4.5 using citric acid). The pH was measured at the beginning of the experiments but was not monitored throughout. We have observed a small drift in pH of less than 1 unit over the course of 48 hrs. Even with this drift, the pH of the bulk solution stayed within the range suitable for fermentation and well-below the pH optimum of XI (pH 7.5). In experiments with co-immobilized pellets, urea concentration was 0, 0.01, or 0.1 M.

Analytical Techniques and Data Analysis To analyze experiments for xylose and xylulose concentration, a 200-μl sample was collected at each time point. The sample was diluted 1:3 with deionized water and then filtered through a 0.2 μm filter. Xylose and xylulose calibration standards with concentrations ranging from 0.25 to 80 g/l in pH 4.5 citrate buffer were prepared in a similar manner. All standards and samples were analyzed by high performance liquid chromatography (HPLC) using a 30-μl injection volume with a 100-μl injection loop. The HPLC unit used was a Shimadzu Series 10A HPLC unit equipped with a SIL-10Ai autosampler and a refractive index detector (RID 10A). A Bio-Rad Aminex HPX-87 P (300×7.8 mm) ion exchange column was used for sugar analysis using a mobile phase of deionized water with a flow rate 0.6 ml/min and a temperature of 80 °C. This column was successful in separating xylose and xylulose. To determine if the xylulose–borate complex dissociated and eluted separately, solutions of borate and borate with xylulose were injected, and the area of the borate peak was measured. As the height of the borate peak was independent of xylulose concentration, we concluded that the xylulose–borate complex dissociated into xylulose and borate, and the xylulose peak represented total xylulose in the mixture. Finally, data for xylose and xylulose concentration at each time point were summed and normalized to 60 g/l total concentration to eliminate variability and to close the mass balance. All xylulose concentrations reported in the presence of borate represent the total xylulose (free xylulose + xylulose-borate complex) concentration. All experiments were performed in duplicate, and data were very reproducible; data shown is representative of one run. Figures were generated in Origin 7.0 (Northampton, MA, USA).

Results and Discussion

The experimental results are organized as follows. The first set of experiments was designed to demonstrate the ability of the co-immobilized pellet system to achieve two

different pH microenvironments within a single vessel—one optimal for XI activity and the other suitable for conducting fermentation. After these experiments, the ability of sodium tetraborate decahydrate (borax) to alter the kinetics and shift the xylose/xylulose equilibrium was investigated in the unaltered and co-immobilized pellet systems. Next, the mass of co-immobilized enzyme pellets was changed, and the effect on the kinetics and approach to equilibrium for the xylose isomerization are presented. Finally, the effect of added metal ions on the isomerization are presented and discussed.

Demonstration of the Sustainability of Two-pH Environments in a Single Vessel

Unaltered Pellets As an initial control experiment, the isomerization of xylose to xylulose was studied using Sweetzyme™ pellets, as received, before co-immobilization with urease. The time course of xylose consumption and xylulose formation was monitored for an initial xylose concentration of 60 g/l with 0.13 g pellets at 34 °C. The isomerization mixture was buffered at pH 7.5, which is the optimal pH for XI activity. As seen in Fig. 2, curve A, the concentration of xylulose steadily increased and reached an equilibrium value of about 9 g/l, suggesting an equilibrium xylose/xylulose ratio of nearly 6:1 under these conditions. When the same experiment was repeated at a reduced pH of 4.5, no xylulose was detected in the reaction mixture, even after 40 h (data not shown). At a pH of 4.5, XI is 3 pH units below its optimum and displays essentially no activity.

XI/Urease Co-immobilized Pellets We next modified the SweetzymeTM pellets by adsorbing urease onto the pellets. The co-immobilized enzyme pellets (0.13 g) were added to reaction media containing 60 g/l xylose buffered to pH 4.5. As with the unaltered pellets, no xylulose formation was observed under these conditions even after 48 h (see Fig. 2, curve C). Next, 0.01 M urea was added to the bulk solution buffered to a pH of 4.5. Formation of xylulose was observed in the presence of urea, and the concentration of xylulose in the reaction medium gradually increased to reach a value of about 5 g/l by 48 h (see Fig. 2, curve B).

The production of ammonia by urea hydrolysis catalyzed by immobilized urease in the pellets raises the internal pH within the core of the pellets as shown in Fig. 1. The interior

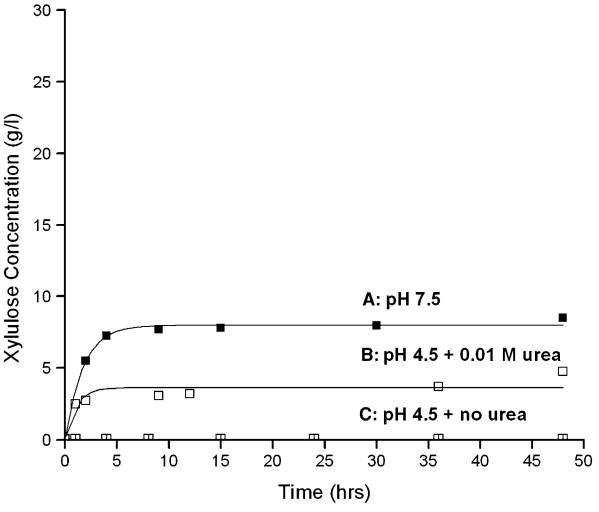


Fig. 2 Proof-of principle that two pH microenvironments are developed in the co-immobilized enzyme system via urea hydrolysis. *Solid symbols* are used for unaltered Sweetzyme™; *open symbols* are used for the XI/urease co-immobilized pellets. The three experiments shown are *A* pH 7.5; *B* pH 4.5 with 0.01 M urea, and *C* pH 4.5 with no urea; each used 0.13 g pellets. Unaltered Sweetzyme™ yielded no xylulose production at pH 4.5 (data not shown). Xylulose production shown for *B* indicates that XI has activity when urea is added

pH must be well above the bulk pH of 4.5 for the XI within the pellets to be catalytically active. Therefore, when the xylose in the bulk solution diffuses into the pellets and reaches a higher pH region where XI is active, xylose isomerizes to form xylulose. At the same time, the continuous production of ammonia in the outer layer (Zone 1, Fig. 1) of the co-immobilized pellets also tends to neutralize any hydrogen ions that diffuse into the core of the pellets from the bulk solution, thereby sustaining the pH difference between the interior of the pellets and the external solution.

Because the rate of isomerization in curve B is lower than that obtained at pH 7.5 in unaltered pellets, it suggests that the interior pH is not maintained at 7.5 but at a suboptimal pH, either above or below 7.5. If the interior pH is suboptimal, then the XI activity will be lower than that in the unaltered pellets at pH 7.5, and we expect that the time required to reach equilibrium will be longer. If XI activity is reduced in the co-immobilized pellets, the time required for isomerization may ultimately exhaust the urea from the bulk solution, at which point XI activity will be lost, and the isomerization reaction will cease.

The interior pellet pH is a function of the urease loading and the urea concentration profile in the pellet. The K_m for urease hydrolysis of urea is 2.9 mM, [29] so with 0.01 M (10 mM) urea, we are initially consuming urea at approximately 78% of V_{max} at the surface of the pellet. Increasing the bulk concentration of urea will result in increased ammonia production and an increase in the interior pellet pH. Depending upon whether the interior pH is above or below the pH for optimum XI activity, an increase in interior pH will decrease or increase the rate of xylose isomerization. To achieve optimal isomerization in the co-immobilized pellet system, the urea concentration in the bulk solution can be optimized for a specific urease loading and should be maintained at a constant concentration throughout the isomerization to allow maximal, constant XI activity.

Although our system has not been optimized to achieve the most favorable internal pH for XI activity, we are able to demonstrate significant XI activity in our co-immobilized enzyme pellets at a bulk pH of 4.5 with 0.01 M urea. As the overall production rate of ethanol is limited by the total concentration of xylulose available to the yeast [9], it is important to determine if we can modify our experimental conditions to favorably enhance the isomerization and the xylose/xylulose proportions. Hence, the next experiments focus on addition of borate to the reaction medium in an effort to enhance the isomerization kinetics and favorably shift the equilibrium. Results for both unaltered and co-immobilized enzyme pellets are presented.

Effect of Sodium Tetraborate Addition on Xylose Isomerization

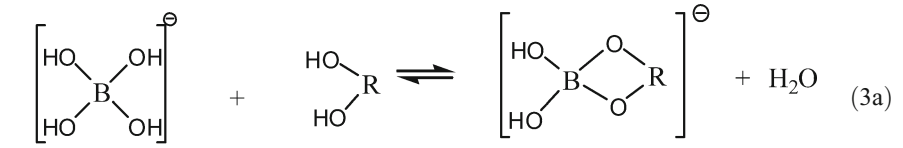
Mechanism of Sugar–Borate Complexation It has been suggested that borate leads to a shift in the equilibrium isomerization because of the binding of tetrahydroxyborate ions to aldose and ketose sugars. At near neutral pH, tetrahydroxyborate ions can be formed by hydrolysis of borax ($Na_2B_4O_5(OH)_4{\cdot}8H_20$) [44]:

$$B_4O_5(OH)_4^{2-}+5H_2O \leftrightarrow 3B(OH)_3+B(OH)_4^-+OH^- \quad (1)$$

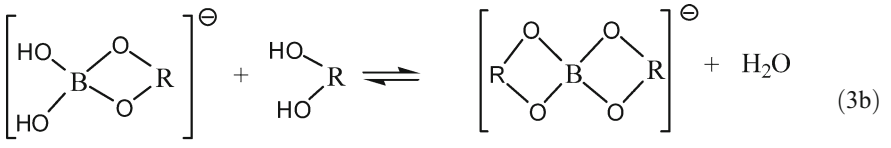
The boric acid produced in the above reaction is a weak-acid (p*K*a~9) that ionizes to a slight extent by reaction with water at neutral pH to form additional tetrahydroxyborate ions:

$$B(OH)_3+H_2O \leftrightarrow B(OH)_4^-+H^+ \quad (2)$$

The tetrahydroxyborate ions produced in the above reactions are able to complex with adjacent hydroxyls on sugar molecules. As shown in Eqs. 3a and 3b, each tetrahydroxyborate ion can bind up to two molecules of sugar in a two-step process.



Tetrahydroxyborate ion **Sugar** **Mono-complex**



Monocomplex **Sugar** **Di-complex**

Borate is able to complex, via the above mechanism, more readily with the open-chain structure of xylulose as compared to the cyclic hemiacetal form of xylose [44]. This binding preference leads to a shift in the xylose/xylulose isomerization equilibrium in favor of xylulose formation.

Unaltered Pellets First, the effect of sodium tetraborate on the kinetics and equilibrium of isomerization for unaltered XI pellets in a buffer of pH 7.5 was studied. These data are shown in Fig. 3, curve A. When compared with the corresponding data obtained in the absence of borate (Fig. 2, curve A), it is clear that even at this low concentration (0.05 M), borate is able to shift the equilibrium significantly in favor of higher xylulose production. The equilibrium concentration of xylulose reaches~30 g/l, which is more that three times

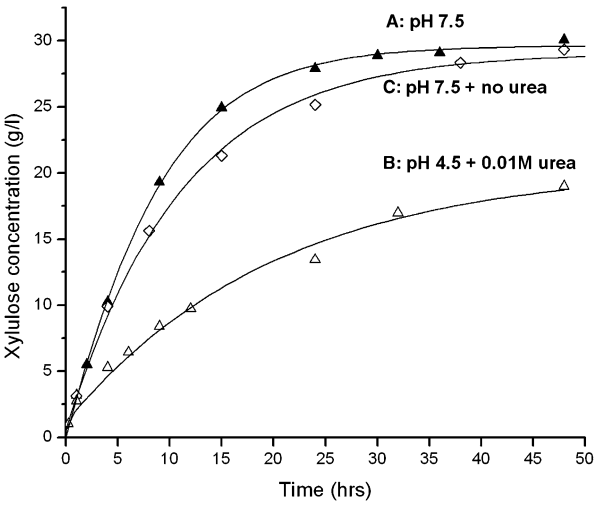


Fig. 3 Borate is able to favorably shift the xylose/xylulose equilibrium for both unaltered and co-immobilized enzyme pellets. *Solid symbols* are used for unaltered Sweetzyme™; *open symbols* are used for the XI/urease co-immobilized pellets. The three experiments shown are *A* pH 7.5; B pH 4.5 with 0.01 M urea, and *C* pH 7.5 with no urea. All three experiments show a significant shift in the equilibrium toward xylulose production. However, only the conditions represented by curve *B* are conducive for simultaneous isomerization and fermentation

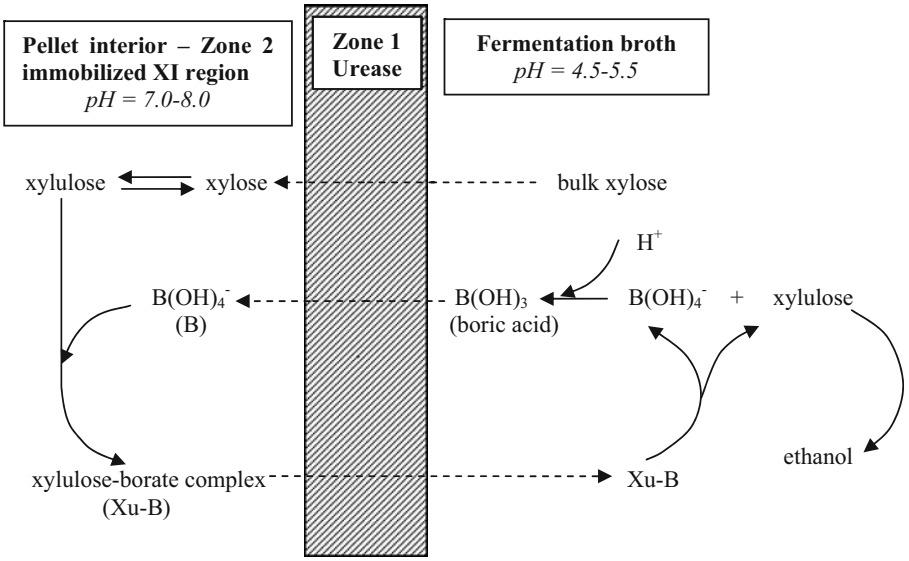


Fig. 4 Role of xylulose–borate complexation in the co-immobilized enzyme system. When sodium tetraborate (borax) is added to solution, it dissociates into tetrahydroxyborate (borate, *B*) ion and boric acid. In the pellet interior, higher pH favors tighter xylulose–borate binding, which effectively reduces the xylulose concentration in the interior and forces the isomerization forward. In the bulk, the lower pH has an uncoupling effect on the Xu–B complex, making the dissociated xylulose readily available to the yeast. Removal of xylulose via fermentation further forces dissociation of the xylulose–borate complex. *Dashed lines* represent transport of species; *solid lines* represent reactions

that seen without borate (~9 g/l). Borate addition leads to an increased conversion of xylose and a shift in the equilibrium xylose/xylulose ratio from about 6:1 to about 1:1.

Urease Co-immobilized Pellets The effect of urease immobilization on the pellets has a negligible impact on the overall kinetics and equilibrium achieved at pH 7.5 as shown in Fig. 3, curves A and C (both run without urea). The immobilized urease may add a small mass transfer resistance, which could account for the slowing of the kinetics seen as the xylose concentration decreases. Next, upon adding urea (0.01 M) to the citrate buffer solution, we again see significant formation of xylulose with our co-immobilized pellet system, with xylulose reaching a concentration of~17 g/l by 48 h. This value is much higher than the corresponding level reached without borate addition, which was about 5 g/l (see Fig. 2, curve B, and Fig. 3, curve B).

As suggested by reactions given in Eqs. 1 and 2, the formation of tetrahydroxyborate ions is affected by the pH of the medium, and consequently, the ability of borax to shift the xylose/xylulose isomerization equilibrium is also a function of pH. At low pH (4 to 5), very few tetrahydroxyborate ions are formed (as the second reaction does not occur) and accordingly borax is less likely to have any influence on the isomerization equilibrium. On the other hand, in the higher pH range (6 to 8), the tetrahydroxyborate ion concentration reaches appreciable levels, and these ions bind strongly to xylulose (Eq. 3), shifting the isomerization equilibrium.

As shown in Fig. 4, in our two-pH environment system, we expect that the core region of the pellets (where the pH is high and XI is active) provides conditions conducive to

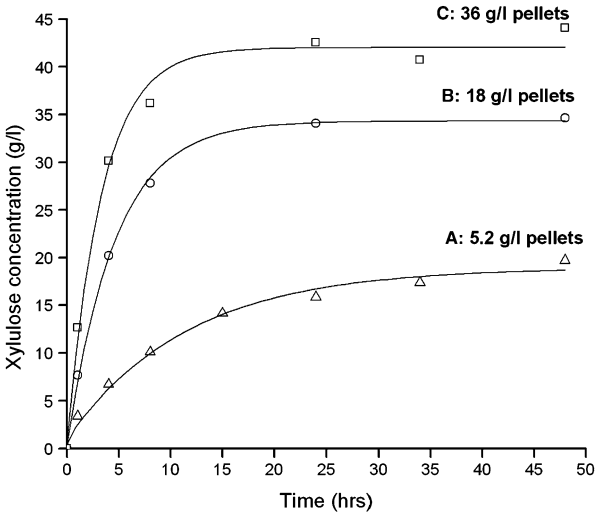


Fig. 5 Effect of XI/urease activity on the isomerization kinetics and xylose/xylulose production for the co-immobilized enzyme pellets. All pellets were from the same co-immobilization batch and have the same urease and XI activities per gram of pellet at pH 7.5. The initial urea concentration used in all experiments was 0.01 M. The improvement in the xylulose yields with increased enzyme loading can be attributed to the dual role of tetrahydroxyborate ions in our co-immobilized pellet system

strong binding of xylulose to tetrahydroxyborate ions and formation of the xylulose–borate complex (Xu–B). However, in the bulk solution where the pH is low, very little borate–sugar complex formation takes place. The likely net result of this two-pH environment in the context of SIF is that boric acid diffuses into the pellets, is converted to tetrahydroxyborate ions (Eq. 2), binds to xylulose, and ferries xylulose from inside the pellet to the bulk solution outside. In the low pH bulk solution, the Xu–B releases xylulose and the borate ions recombine with hydrogen ions to form boric acid. Thus, tetrahydroxyborate, in addition to shifting the isomerization equilibrium, facilitates the removal of xylulose from the core of the pellets into the bulk where xylulose can be readily metabolized by yeast to ethanol. Xylose feed solutions isomerized in the presence of 0.05 M borate have been used in fermentation studies with yeast, and no inhibition of yeast by borate have been observed [45].

Effect of Co-immobilized Pellet Mass on Isomerization

The activity of the Sweetzyme™ pellets co-immobilized with urease depends on many factors [29]. These factors include the concentration of urea and the pH in the bulk solution and the activity of urease immobilized in the outer layer (Zone 1) of the pellet. These factors influence the production of ammonia and the neutralization of the diffusing hydrogen ions and hence the size of the active XI zone (Zone 2).

In Fig. 5, transient xylulose production is shown as a function of total co-immobilized pellet mass. All pellets used were from the same co-immobilization batch and have the same urease and XI loadings. Experiments were conducted at 34 °C and pH 4.5 with 0.01 M urea, 0.05 M sodium tetraborate, and an initial xylose concentration of 60 g/l. Experiments shown in curves B and C have 3.3 (18 g/l) and 6.6 (36 g/l) times more of each enzyme compared to curve A (5.2 g/l). At time zero in all experiments, the interior pH increases rapidly to values closer to the optimum for XI activity as ammonia is produced. In experiments B and C, the increased mass of urease and XI will cause a more rapid decrease in the bulk urea and xylose concentrations than in A. As the bulk urea concentration decreases, the ammonia production per pellet decreases and the interior pH also starts to decrease. This drop in pH occurs earlier in cases where the total urease mass (activity) is higher, leading to an accompanying loss in specific XI activity. From the data shown in Fig. 5, the average specific XI activity was

Table 1 Effect of co-immobilized pellet mass on isomerization kinetics and xylulose production.

Experiment	Mass of pellets (g)	Ave. XI activity in first hour (U)	Specific XI activity in first hr (ave; U/g pellet)	Final [Xylulose] (g/l)
A	0.13	7.8	58.5	20.0
B	0.45	20.4	45.3	34.4
C	0.9	34.9	38.8	44.0

Total XI is proportional to the pellet mass, but the XI activity measured over the first hour depends on the internal pH profile within the pellet. As pellet mass increases, the bulk urea concentration decreases more rapidly and the changing internal pH profile results in an apparent decrease in specific XI activity. Although urea consumption and loss of XI activity occurs most rapidly for the highest pellet mass, the total xylulose produced while the XI is active is the greatest.
1 U=1 μmol of xylulose produced per minute at 34 °C and bulk pH of 4.5.

calculated for the first hour of isomerization; these results are summarized in Table 1. The average specific XI activity (based on xylulose production per g of pellets at pH 4.5) decreases with increasing pellet mass. However, the corresponding total XI activity is higher, resulting in a much more rapid production of xylulose and much higher xylulose yield by 48 h.

It is noteworthy that the xylulose concentration (~44 g/l) at 48 h for the highest pellet mass is substantially higher than the value achieved (~30 g/l) with unaltered pellets at pH 7.5 with the same borate concentration (Fig. 3 curve A). For an unaltered Sweetzyme™ pellet, the kinetics of the isomerization depend on the XI activity, but the equilibrium is governed solely by the thermodynamics and is unaffected by the XI activity and pellet mass. In our co-immobilized pellet system, we are seeing xylulose conversions that are higher than those possible with the unaltered pellets at pH 7.5. As mentioned in the context of Fig. 3 and illustrated in Fig. 4, in our co-immobilized pellet system, tetrahydroxyborate acts to shift the equilibrium by binding to xylulose and also shuttles complexed xylulose from the pellet interior to the bulk solution. We believe that this dual role of tetrahydroxyborate is responsible for the significant improvement in xylose conversion seen in our co-immobilized pellet system when a pH gradient is established.

Effect of Urea

The urea concentration in the bulk media will affect the rate and quantity of ammonia produced and hence the maintenance of the pH gradient within the co-immobilized enzyme pellet. The urea concentration will also determine the volume of the active XI core, and this will in turn influence the kinetics of the isomerization and the extent of isomerization. In Fig. 6, transient xylulose production is shown as a function of urea concentration. All pellets used were from the same co-immobilization batch and have the same urease and XI loadings. Experiments were conducted at 34 °C and pH 4.5 with either 0.01 M (curve A) or 0.1 M urea (curve B), 0.05 M sodium tetraborate, and an initial xylose concentration of 60 g/l.

As seen in these the two experiments, the rate of xylose isomerization is very similar for the first 4 h. For both cases, the concentration of urea is significantly higher than the K_m for urease so the internal pH profiles within the pellet are likely to be similar. As the pellets also have the same XI loading, xylulose production is equivalent in both. However, by 8 h, urea consumption in A results in a decrease in reaction velocity for urea hydrolysis. With reduced ammonia production, the internal volume of the pellet with active XI decreases, and a drop in xylulose production relative to B is observed. Based on the results shown for A, urea hydrolysis is no longer effective at maintaining the two pH microenvironments by 24 h.

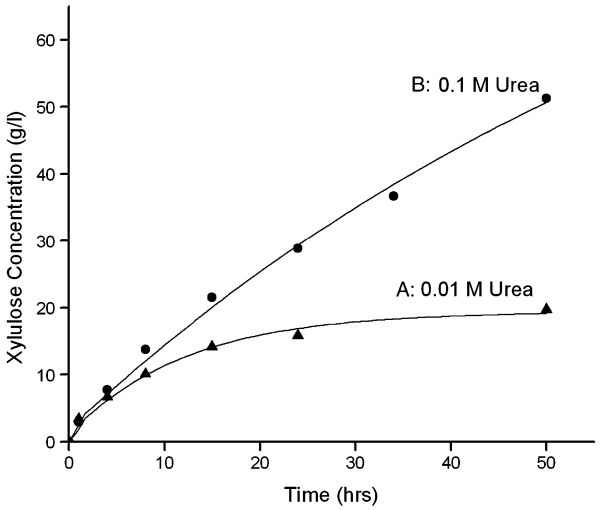


Fig. 6 Effect of initial urea concentration on the isomerization kinetics and xylulose production for the co-immobilized enzyme pellets. Both experiments use 0.13 g pellets from the same co-immobilization batch and have the same urease and XI activities per gram of pellet at pH 7.5. The decrease in the rate of isomerization and xylulose production seen in *A* is because of consumption of urea. Urea concentration is initially higher than the K_m for urease, but as the urea concentration drops, the reaction velocity and ammonia production decrease, resulting in a loss of the pH gradient within the pellet. In *B*, the urea concentration is high enough that the rate of xylulose isomerization does not appear to be affected by urea consumption over the entire 48-h period

For B, with a much higher initial urea concentration, the active zone for xylose isomerization is maintained for a much longer period of time (>48 h). The final xylulose concentration measured at 48 h was~52 g/l, corresponding to a xylose/xylulose ratio of ~1:6.5.

Effect of Co-immobilized Pellet Mass on Isomerization in Presence of Excess Urea

The effects of pellet mass and urea concentration on the final composition of the isomerization solution are summarized in Fig. 7. Pellet mass ranged from 0.13 to 0.9 g per experiment, whereas the initial urea concentrations were either 0.01 or 0.1 M. For 0.01 M urea (B and C), the increase in pellet mass results an increase in rate of xylulose production (see also Fig. 5) as well as an increase in the total xylulose produced. However, none of the experiments with 0.01 M urea reach a xylulose yield as high as that achieved when 0.1 M urea is added. For 0.1 M urea (D and E), the increase in pellet mass also results in an increase in the rate of xylulose production and a reduction in the time required to reach the final solution composition, but the final xylulose yields remain unchanged. Although increasing the pellet mass (more XI) increases the isomerization kinetics, urea plays an essential role in maintaining XI activity and achieving high xylulose yields. The co-immobilized enzyme system, by virtue of the unique two-pH microenvironments and the borate shuttling of xylulose to the bulk, results in conversion of xylose to xylulose (~86%) that is significantly higher than that achievable with the native XI at its optimal pH.

Effect of Metal Ion Addition on Xylose Isomerization

In addition to evaluating the effectiveness of borate in favorably shifting the xylose to xylulose equilibrium, we were also concerned with maintaining sustained optimal activity of

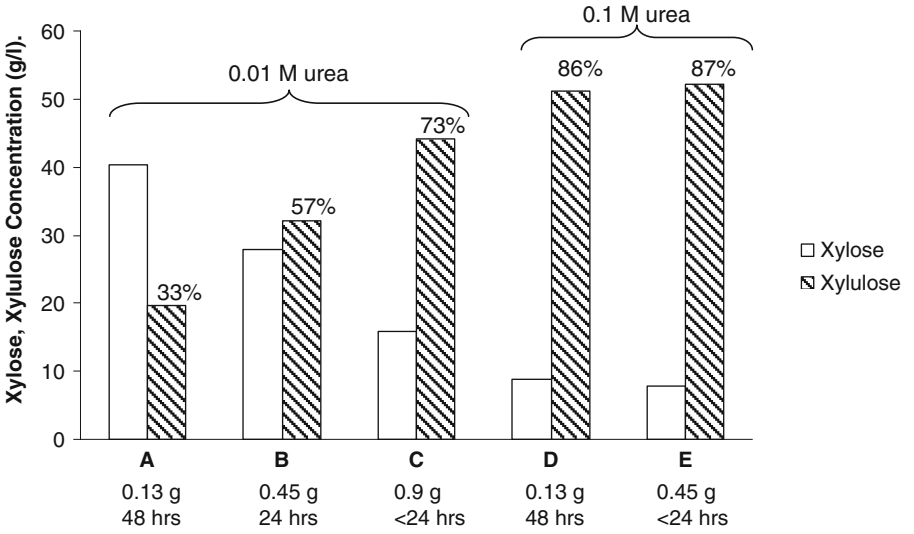


Fig. 7 Effect of initial urea concentration and mass of pellets on xylulose production for the co-immobilized enzyme pellets. All pellets were from the same co-immobilization batch and have the same urease and XI activities per g pellet at pH 7.5. The percentage conversion to xylulose is given *above the bars* for each experiment. The time indicated on the *x*-axis represents the time of apparent equilibrium for all experiments. With 0.01 M urea (*A*, *B*, and *C*), increasing the pellet mass results in a significant increase in xylulose formation. With 0.1 M urea (*D* and *E*), the final xylulose production is only minimally increased by increasing the mass of pellets

XI for long time periods. The XI enzyme requires metal ions for activity, and these ions can be depleted during the isomerization [46]. It has been suggested that improvement in long-term activity of XI can be realized by the addition of Mg^{2+}, and Co^{2+} ions to the medium [47, 48].

In these experiments, unaltered Sweetzyme™ at pH 7.5 was used. In the absence of borate, addition of metal ions results in a small shift in the isomerization toward xylulose (Fig. 8, curves A and B). In the presence of borate, a similar shift in the isomerization is

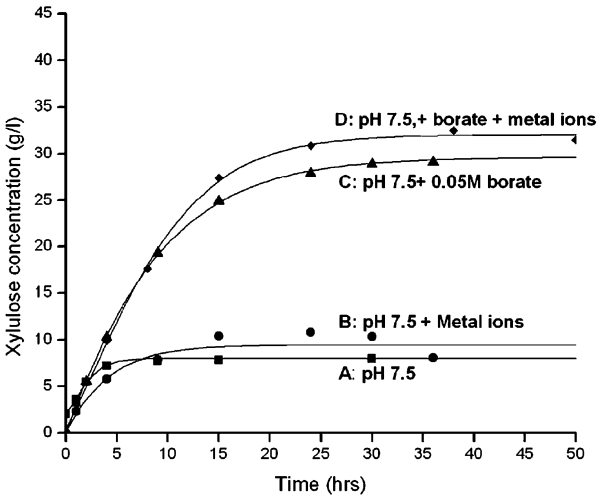


Fig. 8 Addition of metal ions results in a small shift in the isomerization toward xylulose, but the effect is not as significant as the shift associated with addition of sodium tetraborate. If added, sodium tetraborate was 0.05 M, and metal ions were 20 mM $MgCl_2$ and 1 mM $CoCl_2$. All data shown are for unaltered Sweetzyme™ at pH 7.5. The four experiments shown differ by additives and are *A* no additives, *B* metal ions, *C* borate, and *D* borate and metal ions

observed (Fig. 8, curves C and D). Thus, metal ions either alone or in conjunction with borate provide an incremental improvement in the xylulose production, but the effect is not as significant as the shift associated with addition of sodium tetraborate.

Summary and Conclusions

One of the crucial issues in ethanol production from lignocellulosic biomass is the ability to convert both the hexose (C6) and pentose (C5) sugars resulting from the saccharification of its cellulose and hemicellulose portions. In this paper, a method for producing high yields of xylulose from xylose isomerization (the principal C5 sugar from hemicellulose) is presented, as xylulose can be converted to ethanol by native *S. cerevisiae* along with glucose (C6 sugar).

In our experiments at pH 7.5 and 34 °C with SweetzymeTM pellets, we found that starting with 60 g/l of xylose; we could shift the equilibrium concentration of xylulose from~9 to~30 g/l by the addition of 0.05 M borax. The positive shift in equilibrium results from the selective complexation of xylulose to the tetrahydroxyborate ions formed from borax at this pH. Although the xylulose production is significantly enhanced with borate, if the isomerization and the fermentation steps are sequential, only half of the available xylose may be converted to ethanol.

Simultaneous isomerization and fermentation (SIF) is expected to drive the isomerization forward and lead to higher xylose utilization. Unfortunately, the pH optima for the isomerization (~7.5) and the fermentation (~4.5) steps are vastly different. The approach we have taken is to develop a novel technique for SIF that is capable of sustaining two different pH-microenvironments in a single vessel—one optimal for xylose isomerization and the other optimal for fermentation of xylulose. The technique involves co-immobilization of urease with xylose isomerase. We have shown that it is possible to sustain a significant pH gradient between the bulk liquid and the core region of the pellet by adding urea to the fermentation broth.

Using our co-immobilized enzyme system in media supplemented with borate, we have obtained xylose conversions that are higher than those possible with the native XI pellets operating under optimal pH at the same borate concentration. The advantage of the co-immobilized enzyme system results from the pH-dependent nature of borate/xylulose binding, and Fig. 4 summarizes our hypothesis as to why isomerization is enhanced in our system. The results presented demonstrate the effectiveness of our co-immobilized enzyme system for isomerization under conditions optimal for fermentation by common *S. cerevisiae*. As the overall production rate of ethanol in SIF is limited by the total concentration of xylulose available to the yeast [9], our technique significantly improves upon currently available options for SIF.

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