STRATEGIES TO RECYCLE ENZYMES AND THEIR IMPACT ON ENZYMATIC HYDROLYSIS FOR BIOETHANOL PRODUCTION

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Enzymes still exhibit activities after hydrolysis of biomass according to previous studies. Recycling the enzymes and use them in subsequent hydrolysis cycles can further utilize their remaining activities. Previous studies have mainly discussed enzyme recycling processes up to three cycles, in which the processes did not reach steady state. Steady state investigation is essential for the guidance of the real life process. Four cycles of processing have usually been considered enough to bring the system to steady state in process engineering. In this work, hydrolysate was used as the source of recycled enzymes to fresh substrate for five cycles. Because a large amount of enzymes remained on the pulp, surfactant was introduced to recycle the enzymes that remained with the residue. Recycled hydrolysate from previous enzymatic hydrolysis usually carries a high concentration of sugars, which can inhibit the new round of hydrolysis. To remove sugar from the recycling stream, a wash with fresh buffer was performed. Sugars were removed, while enzymes still remain on the fresh substrates. Six recycling strategies were evaluated for enzyme recycling percentage and enzymatic hydrolysis efficiency with both green-liquor pretreated softwood and hardwood in this investigation. Hydrolysis efficiency increased by about 40% for softwood at 30 mg/g enzyme dosage and about 25% for hardwood at 7.5 mg/g when a washing stage was applied with addition of surfactant.

Keywords: Enzyme recycle; Woody biomass; High consistency; Biomass to ethanol; Process development

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INTRODUCTION

The importance of sustainable fuels and chemicals derived from lignocellulosic biomass has become critical because of decreasing fossil fuel inventory and increasing energy demand (Huber et al. 2006; Han et al. 2011). The Environmental Protection Agency (EPA) has set an ambitious target of 3.45-12.9 million gallons (BG) cellulosic biofuel production for the year 2012, despite the fact that there is no commercial-scale facility as of the end of 2010 (Gonzalez et al. 2011). There are still some cost-ineffective limitations (e.g., high capital expenditure, high operation cost, etc.) remaining in the biomass-to-ethanol process (Ezhumalai and Thangavelu 2010).

The biofuel production process can be divided into three integrated stages: pretreatment, saccharification, and fermentation. Enzymes cost is one of the major costs of hydrolysis (Tu et al. 2007; Gonzalez et al. 2011). Therefore, a reduction in the cost of enzymes would be essential to make enzymatic hydrolysis more economically feasible (Zacchi et al. 1988; Xu and Chen 2007).

The first step of enzymatic hydrolysis has been defined as the adsorption of hydrolytic enzymes onto the substrate. Enzymes can bind both to reactive or non-reactive substances (such as lignin). However, lignin may irreversibly adsorb enzymes, decreasing their activity towards a reactive substrate. As the saccharification reaction proceeds, a fraction of adsorbed cellulase is gradually released back into the supernatant because of the solublization of substrate (Lee and Fan 1983; Ooshima et al. 1990), while a substantial portion of the enzymes still remains with the residue. For kraft hardwood and softwood, the majority of the CBHs (cellobiohydrolase) and EGs (endoglucanase) could be absorbed by lignin, and these were prevented from resolubilization at the completion of cellulose hydrolysis (Lee et al. 1995; Xu and Chen 2007). Many surfactants can help to release enzymes from non-active binding and thus elevate the recovery of cellulase activity in supernatants (Yang and Wyman 2006; Kristensen et al. 2007; Xu et al. 2008).

Enzymes can remain relatively active for several rounds of recycling, reducing the need for the addition of fresh enzyme during the softwood-based bioconversion processes (Lu et al. 2002; Bajpai 2010). The Langmuir adsorption isotherm predicted that 82% of the free cellulases could be recovered via readsorption onto fresh substrates of an ethanol-pretreated mixed softwood substrate. The addition of surfactant increased the free enzymes in the supernatant from 71% of the initial protein to 96% (Tu et al. 2007; Steele et al. 2005). Also, adding fresh cellulases may "restart" the slowed-down hydrolysis due to the lack of available, active enzymes (Eriksson et al. 2002).

Since enzyme cost is one of the major contributions to the overall cost of biomass hydrolysis, and recycled enzymes are still active, recycling of enzymes can be an effective way of reducing the cost for enzymes. In previous enzyme recycling investigations, only up to three cycle processes were investigated, in which circumstances, the processes still did not reach the steady state. Steady state investigation is crucial for evaluation of the real life process. Four cycles usually have been considered enough to bring the system to steady state in process engineering. In this work, hydrolysate was used as the source of recycled enzymes to fresh substrate for five cycles. However, recycled hydrolysate usually contains a high concentration of sugar, and the activity of new enzymes can be inhibited. The object of this work is to develop and evaluate enzyme recycling strategies that could eliminate the sugar inhibition problems. Furthermore, a substantial proportion of enzymes still remains on solid residue after hydrolysis in the absence of surfactants; such enzymes could not be recycled, so surfactant was used in this work to improve the amount of recycled enzymes. To relieve the sugar inhibition effect, filtration and a wash step were introduced in this study; this approach mimics a commercial wet press and washer. Six recycling processes were developed, and their enzyme recycling rate, along with enzymatic hydrolysis efficiency, were evaluated using green-liquor pretreated softwood and hardwood.

EXPERIMENTAL

Substrate Preparation

Mixed hardwood and softwood chips were obtained from a mill in the southeastern United States. After screening with 3/8'' and 5/8'' holes, accepts in this range were used

for green liquor (GL) pretreatment. The pretreatment was conducted at 160°C in a 7 liter M&K digester with 800 OD grams of wood chips. Green liqour, which is comprised of Na_2S and Na_2CO_3, was charged at 16% TTA (total titratable alkali) as Na_2O. Contents were pulped to a target H factor at 800. Pulping conditions are summarized in Table 1. After pulping, the pulp was washed overnight and then disintegrated with a refiner at a 0.005 inches gap and a 0.008 inches screen plate. The rejects then were refined again with a 0.001 inches gap, and added back to the accepts. Pulp was centrifuged and fluffed for further processing (Jin et al. 2010).

Table 1. Pretreatment Condition of Wood Chips for Green Liquor Pretreated Pulp

Parameter	Condition
TTA [a]	16%
Sulfidity [b]	25 %
H Factor [c]	800
Liquor to Wood ratio	4:1
Cooking Temp	160°C

[a] TTA: Total of all viable sodium alkali compounds. Calculated as Na_2O based on bone dry wood chips.
[b] Sulfidity was defined as ratio of Na_2S to TTA.

Raw Material Analysis

0.1 grams of pulp was air dried for 24 hours and added to 1.5 mL of 72% sulfuric acid. Pulp was stirred every 15 minutes for 2 hours and transferred into a serum bottle with 56 mL of deionized water. The sealed serum bottle was heated to 120°C at 1.25 atm for 90 minutes (including 30 minutes temperature ramping time) (Jin et al. 2010). Autoclaved suspension was filtered with a fine size crucible after cooling down with running tap water. Filtrate was collected for acid soluble lignin content analysis and the residue was oven dried for Klason lignin content analysis. Sugar analysis was also performed on filtrate with Dioniex Ionic Chromatography (IC) system (Table 2).

Table 2. Materials Analysis

	Glu [b]	Xyl	Gala	Man+Ara	Carbo	ASL	Klason	Lignin	Balance
SW[a]	57.66	7.14	1.30	4.67	53.5	0.52	28.90	29.42	98.83
HW	61.05	14.95		0.55	76.55	0.41	18.47	18.88	95.43

*Experiments were duplicated with difference less than 5%.
** Ara=Arabinan; Gala=Galactan; Glu=Glucan; Xyl=Xylan; Man=Mannan, Carbo= Total Carbohydrates; ASL= Acid Soluble Lignin; Klason= Klason Lignin. And all these number are based on the percentage of pulp.
*** Sugar standard solutions were autoclaved with acid hydrolysate samples in the same batch.
a. SW is abbreviated for softwood. HW is abbreviated for hardwood.
b. C6 sugars were converted as monomer content divided by 1.1 while C5 sugars were by 1.136.

Enzymatic Hydrolysis

All enzymatic hydrolysis experiments were performed with Novozymes (Franklinton, NC) cellulase (NS50013), xylanase (NS50014), and β-glucosidase (NS50010) mixed at a volume ratio of 10:3:3, as was recommended by the vendor. 30 mg of fresh enzyme protein was charged for each gram of pulp for each cycle, aside from the enzyme protein recycled back from the previous cycle. A pH 4.8 NaAc-HAc buffering system was used at 100 mM ionic strength. Erlenmeyer flasks containing enzymes and substrate were incubated at 5% solids loading, 50°C, 160 rounds per minute (RPM), for 48 hours for each cycle of reaction. Tween 80, (Polyoxyethylene sorbitan monooleate) from Acros Organics was used as surfactant for some of the hydrolysis processes.

Protein Detection

Protein concentration was measured with Bradford reagent from Sigma Aldrich using Bovine Serum Albumin as the standard, following the Sigma technical report for Bradford reagent. Absorbance was detected with a 595 nm wavelength with PerkinElmer Model Lambda XLS UV (ultraviolet) - visible spectroscopy. The amount of protein added at the beginning of each cycle was reported as the sum of protein adsorbed onto fresh pulp and fresh protein was added into the hydrolysis system. The amount of protein adsorbed onto pulp was calculated as the difference of protein added into fresh pulp from the amount that ends up in the filtrate.

Sugar Analysis

Sugar released during enzymatic hydrolysis was determined with a Dionex ICS-3000 Ionic Chromatography system. Samples were boiled for 5 minutes to denature enzymes and filtered with a Milipore 22 μm membrane before being injected into a Carbo pak- PA 10 column. Ambient temperature was 18°C with the flow rate of 1.1 mL MiliQ water per minute, while the column pressure was around 1800 psi. Eluent was 200 mM NaOH after sugar separation. A standard curve was made with glucose, xylose, mannose, arabinose, galactose, and rhamnose from Sigma. The internal standard was L-Fructose.

Process Description of Recycling Strategies

As illustrated in Figure 1-a, conventional enzymatic hydrolysis was conducted by mixing enzymes with pretreated biomass to the desired total solids in a mixing tank. The contents were incubated, and sugars for fermentation were produced by cleaving polymer sugars in biomass to fermentable monomer sugars with enzymes. The advantage of this process is its simplicity. However, in the conventional process, the enzyme associated with hydrolysate, which demonstrated cellulase activity, could not be utilized again and was considered lost (Tu et al. 2007). The process needs to be modified to both recycle enzymes in hydrolysate and increase sugar conversion by the recycled enzymes.

In Schemes 1 and 2 (Figure 1-b), the enzyme mixture (30 mg enzyme protein per gram of substrate) was added into the pulp to make up a 5% total solids suspension. After 48 hours incubation, the suspension was filtered, and the filtrate (15/19 of the liquor in the hydrolysis could be collected and recycled) was recycled into the new cycle of enzymatic hydrolysis. Fresh enzymes (another 30 mg enzyme protein per gram of pulp) were added aside from the recycled enzymes onto fresh pulp for every cycle.

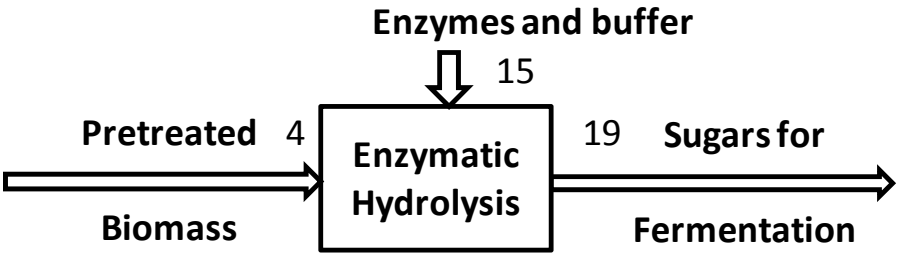

Fig. 1-a. Conventional process for enzymatic hydrolysis for every one part of pretreated biomass (w/w)

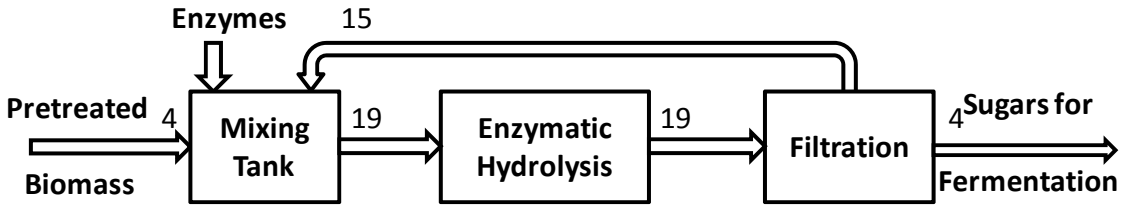

Fig. 1-b. The entire component in the recycling filtrate in this scheme was brought back to the next cycle. (Denoted as Schemes 1 and 2 in the following context. Scheme 1 was performed without surfactant; Scheme 2 was performed with surfactant).

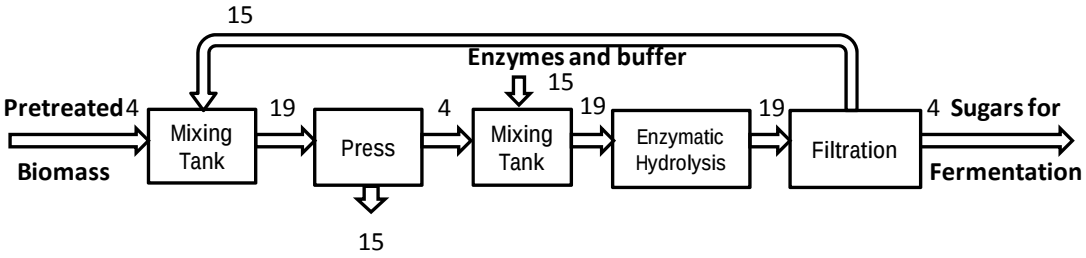

Fig. 1-c. The recycled stream of hydrolysis filtrate was retained in fresh pulp for 10 min and was filtered afterward for the next cycle of enzymatic hydrolysis (denoted as Schemes 3 and 4 in the following context). Scheme 3 was performed without surfactant; Scheme 4 was performed with surfactant.

A process with 0.1% surfactant (based on the total liquid volume of this system) was also conducted with this strategy. Fresh surfactant was added into process at the beginning of each cycle. In this process, sugar and surfactant concentration are going to be built up, which is going to negatively affect the following cycles. Severe end product inhibition would be expected after a couple of cycles of this process.

In Schemes 3 and 4 (Fig. 1-c), filtrate from the previous cycle was recycled (about 15/19 of the liquor in the hydrolysis was collected and recycled) and blended with fresh pulp for 10 minutes. Then the suspension was pressed to 20% solids and fresh enzymes (30 mg enzyme protein per gram of substrate at beginning of each cycle) were added to substrate and make up a 5% total solids suspension. The new cycle of enzymatic hydrolysis was incubated for another 48 hours. In this scheme, sugar concentration in the process would not build up as fast as in Schemes 1 and 2, since some of the sugar could

leave the system at the press and go for fermentation. Enzymes, on the other hand, are expected to stay with the fresh substrate, since they are capable of binding with pulp. However, the sugar concentration is still going to build up gradually and may still introduce end product inhibition to the system after many cycles.

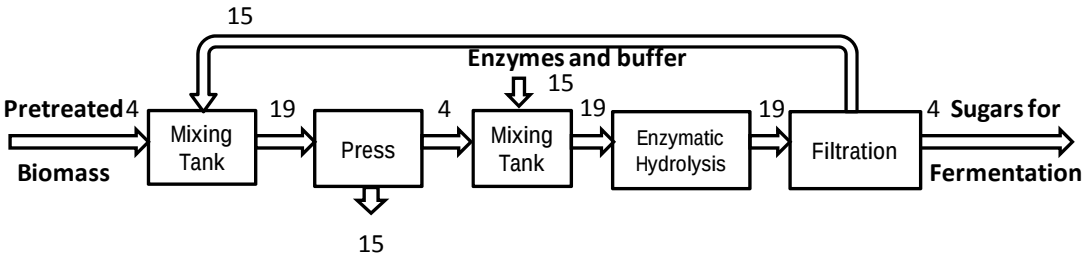

Fig. 1-d. Schemes 5 and 6, enzyme recycling strategy with washing stage (Scheme 5 was performed without surfactant, while Scheme 6 was performed with surfactant)

A washing stage was thus added to the process in Schemes 5 and 6, in contrast to Schemes 3 and 4. After the recycled stream of hydrolysate was pressed out of fresh pulp, buffer was applied to wash pulp with adsorbed enzyme and residual sugars. Washed pulp was added with fresh buffer and enzymes to make up a 5% total solids system that proceeded to the next round of enzymatic hydrolysis. In this scheme, the recycled sugar was expected to be washed away at the washer, while the enzymes will still adhere with the fresh pulp. With this strategy, the sugar concentration is not going to accumulate in process. Aside from the recycled enzymes, 30 mg enzyme protein per gram of substrate was added at beginning of each cycle. The stream from the press is at high sugar concentration and can be subjected to fermentation, while the low sugar concentration from the washer can serve as brown stock washing liquid and thus recycled back into the system, and therefore minimize the loss of enzymes and sugars.

RESULTS AND DISCUSSION

Enzyme Recycling in Softwood Hydrolysis

Enzyme recycling, Schemes 1 and 2

Schemes 1 and 2 were investigated first. The initial enzyme loading was about 30 mg per gram of substrate, and the initial weight loss of hydrolysis was 28 to 30%. The first cycle involves no recycling stream effect and therefore served as the baseline for weight loss and enzyme amount comparison for the following cycles. It is apparent (Fig. 2-a) that Tween 80 significantly increased the amount of recycled enzymes (in which case, about 60 to 80% of enzymes were recycled for the first cycle) compared to the case without adding surfactant (a maximum of 30% enzyme recycling was observed), which indicated that Tween 80 has extensively desorbed the protein that had adsorbed on softwood GL 16 pulp fibers during enzymatic hydrolysis. A high level of enzyme recovery was achieved; however, the case without surfactant resulted in no improvement in weight loss (Fig. 2-b), while the case with surfactant was even worse (up to 50% drop in weight loss was observed) although the enzyme amount accumulated cycle after cycle.

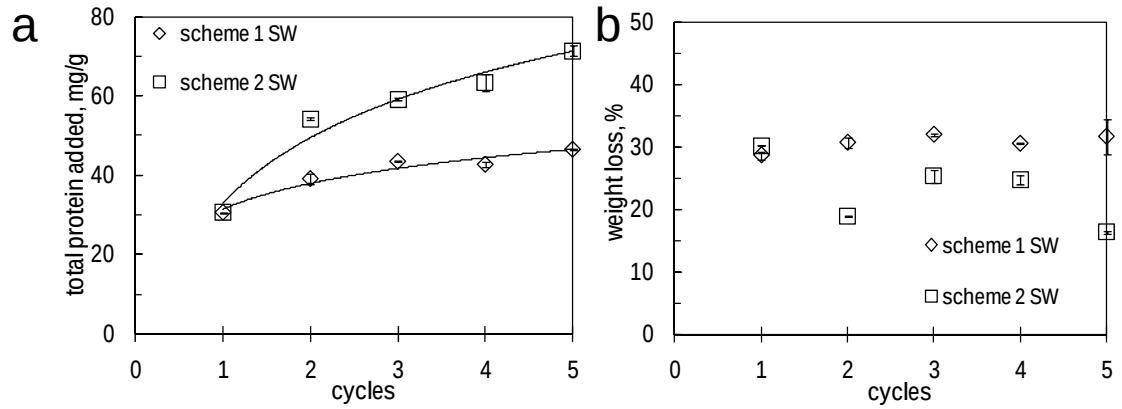

Fig. 2. a. Protein amount at start of each cycle of Schemes 1 and 2 on softwood, and **b**. weight loss data for Schemes 1 and 2. Scheme 1 was performed without surfactant; Scheme 2 was performed with surfactant.

Sugar inhibition and surfactant influence on sugar conversion of softwood

The previous work of Ramos et al. (1993) indicated that increasing sugar concentration can reduce conversion yield of perioxide pretreated eucalyptus, while replacing the liquid phase of enzymatic hydrolysis with new buffer will increase total conversion. It was noticed that during Schemes 1 and 2 that the enzymatic hydrolysis system kept building up sugar and surfactant concentration. Thus, hydrolysis efficiency, which is represented as weight loss, would be inhibited by the accumulation of sugar or surfactant. Sugar and surfactant inhibition was evaluated by adding a specific amount of sugar or surfactant into GL 16 softwood pulp enzymatic hydrolysis system at 30 mg enzyme protein dosage. Research by Ericsson et al. (2002) and Castanon and Wilke (1981) have shown that Tween 80 at 0.1% to 0.25% concentration can have a positive effect on hydrolytic performance. However, the present study showed that the addition of Tween 80 did not result in higher sugar conversion in the investigated range (Fig. 3-b). Tu et al.'s research (2007) also showed little increase in hydrolysis efficiency with addition of surfactant in the case of ethanol-pretreated lodgepole pine. From Fig. 3-a it is obvious that the decreasing trend of conversion ratio was due to sugar inhibition (50% decrease in weight loss in investigated range as shown in Fig. 3-a) rather than surfactant accumulation. The enzyme used in this study seems to be sensitive to sugar concentration increases. Sugar intolerance of these enzymes suggested that it is important to find a way to remove recycled sugar in the consecutive round of hydrolysis, while retaining recycled enzymes on fresh pulp.

Enzyme recycling, Schemes 3 and 4

Schemes 3 to 6 were based on an expected high affinity of hydrolytic enzymes towards substrate, which was first purposed by Sinistyn et al. (1983). In their studies, when the hydrolysate stream recycled back into next cycle was passing through fresh pulp, enzymes would be recovered into the new hydrolytic system by being adsorbed onto fresh substrate.

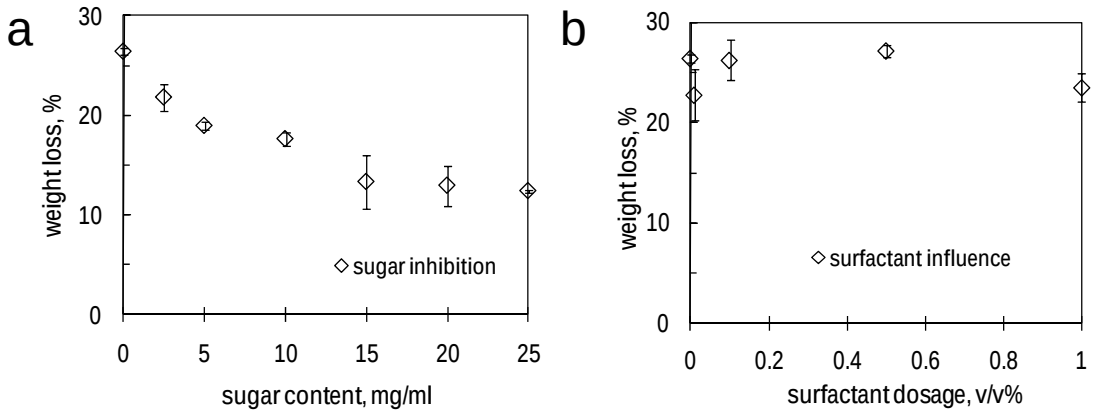

Fig. 3. a. Weight loss data for sugar inhibition effect, and **b**. surfactant inhibition effect

It was suggested that noncellulosic material, e.g. lignin, can nonspecifically adsorb enzymes (Lu et al. 2002), by hydrophobic interaction or ionic interactions (Jorgensen et al. 2007), and therefore has an influence on recycling efficiency. However, Schemes 3 and 4 showed negligible improvement (about 5%) in sugar conversion (Fig. 4) despite the fact that the enzyme amount was increased by 50%.

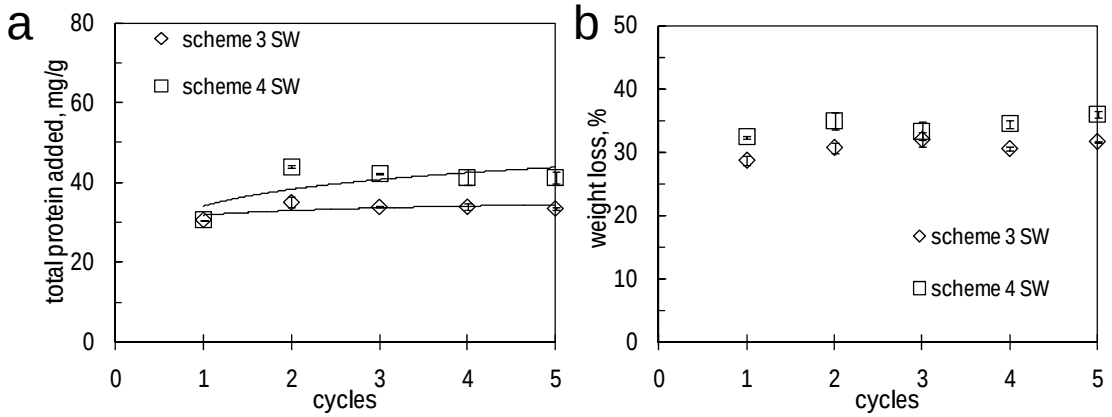

Fig. 4. a. Protein added at start of each cycle and **b**. weight loss data for Schemes 3 and 4. Scheme 3 was performed without surfactant; Scheme 4 was performed with surfactant.

Enzyme recycling, Schemes 5 and 6

Technologies used in Schemes 5 and 6 are proven and involve essentially no risks. Tu et al. (2007) suggested that the fraction of recycled cellulase that can bind with fresh substrate bears the highest activity. Therefore, the washing stage applied in Schemes 5 and 6 is not supposed to wash away much of the adsorbed cellulase activity, but it can remove most of the sugar from recycled hydrolysate. In this way, Schemes 5 and 6 eliminated sugar inhibition, while the recycle enzyme remained on fresh pulp. Figure 5-a shows about 10% of the enzyme amount increase with Scheme 5 and 20% of the enzyme amount increase with Scheme 6. As shown in Fig. 5-b, 31% of substrate was hydrolyzed in 48 hours on the third cycle with Scheme 5, which is 20% higher than the first cycle. In comparison, 40% of substrate was hydrolyzed in 48 hours on the third cycle

with Scheme 6, which is 42% higher than the first cycle, which involved no recycling stream. Ramos et al. (1993) suggested that the components do not equally adhere onto lignocellulose. Instead, biomass selectively adsorbs enzymes onto its surfaces. Recycling the filtrate would lose a particular proportion of enzymes. It is also known that the hydrolysis of biomass needs a group of hydrolytic enzymes to collaborate for efficient conversion. Therefore, the synergistic interaction will be affected. This could be the reason why the case with surfactant showed higher conversion yield than the scenario without surfactant, since Tween 80 can reduce binding and is able to liberate various enzymes into supernatant and thus recycle back to the next cycle.

Probably beta-glucosidase cannot be recycled effectively with the schemes proposed in this article. That is why sugar inhibition becomes significant, because only CBHs and EGs were recycled each time, even the addition of fresh enzyme cannot supplement enough beta-glucosidase to reduce the end product inhibition. That is why washing becomes important in Schemes 5 and 6.

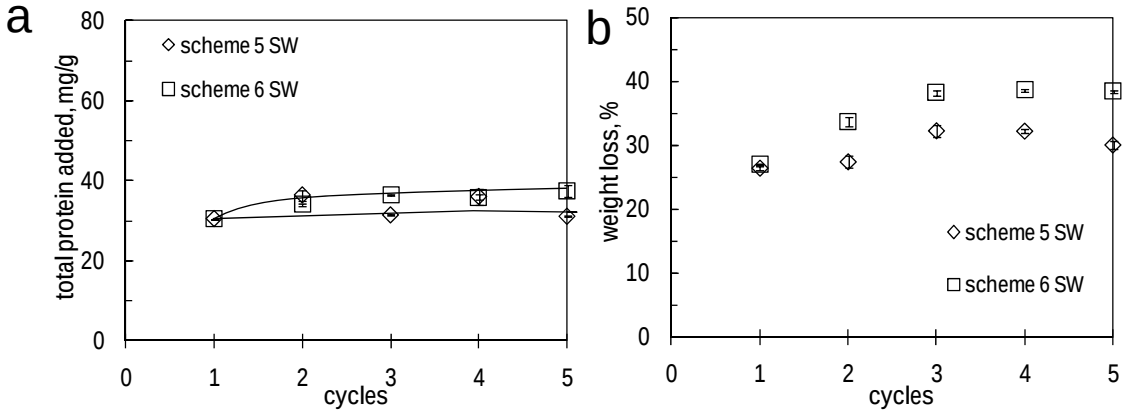

Fig. 5. a. Protein added at start of each cycle and **b.** weight loss data for Schemes 5 and 6. Scheme 5 was performed without surfactant; Scheme 6 was performed with surfactant

Sugar split for Schemes 1 through 6

While all the sugar went for the next cycle of hydrolysis in Schemes 1 and 2 (Fig. 6-a), Schemes 3 and 4 had about 30 to 40% of sugar from previous hydrolysate remaining in the fresh pulp (Fig. 6-b), which still can cause an inhibition effect for the new cycle. The washing stage can wash away most of the sugar that remains with fresh pulp and therefore reduce inhibition from previous released sugar (Fig. 6-c).

Lignin degradation by-products may generate during enzymatic hydrolysis (Kirk and Farrell 1987). Especially when the process was conducted with the addition of Tween 80, the hydrolysate resulted in a much darker color after filtration compared with the filtrates without adding surfactant. Some of the lignin degradation by-products can negatively influence the next cycle of hydrolysis when the concentration was built up to a toxic level (Palmqvist et al. 1996; Sewalt et al. 1997). Filtration and washing stage can also effectively remove soluble lignin degradation by-products generated during enzymatic hydrolysis.

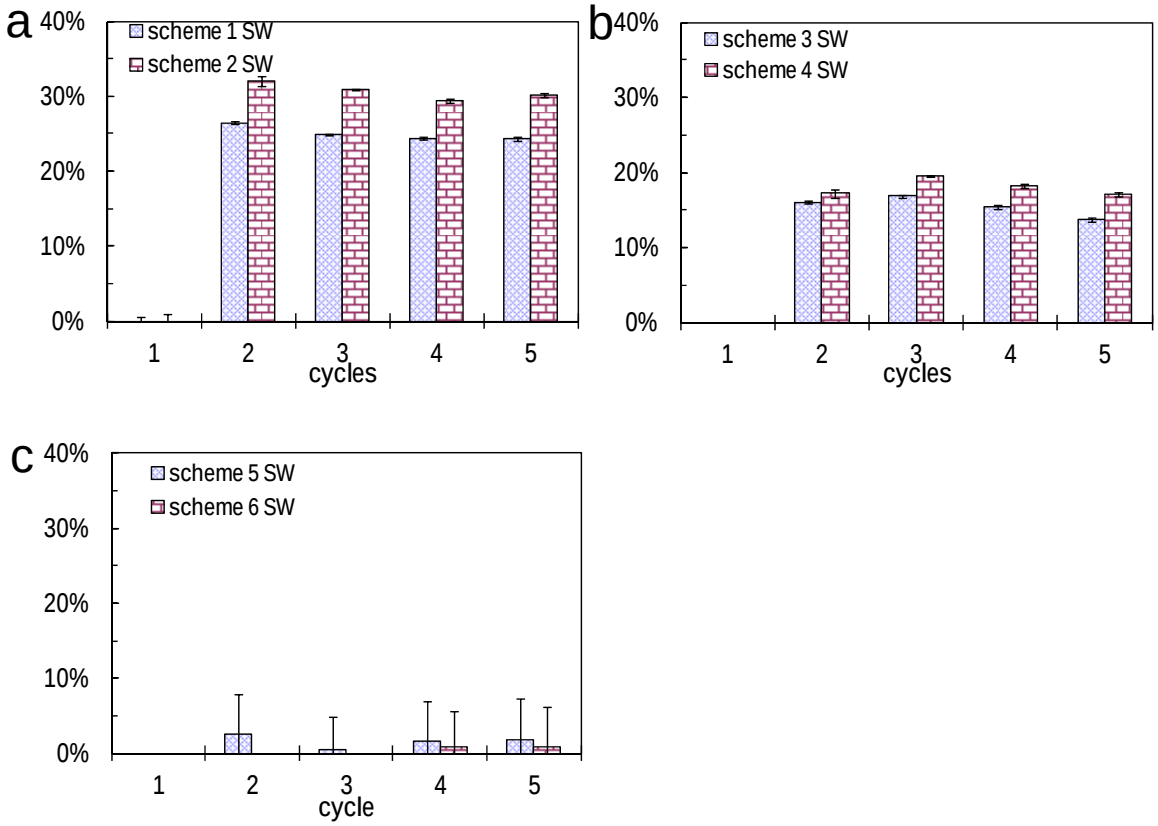

Fig. 6. Sugar percentage remaining in fresh pulp generated from the previous cycle with Schemes 1 through 6. (**a.** Schemes 1 and 2; **b.** Schemes 3 and 4; **c.** Schemes 5 and 6). Schemes 1, 3, and 5 were without surfactant; Schemes 2, 4, and 6 were with surfactant.

Enzyme Recycling in Hardwood Hydrolysis

Schemes 1 through 6 for hardwood enzyme dosage profile

It has been suggested that enzymatic hydrolysis efficiency is significantly influenced by substrate characteristics (Lu et al. 2002). Generally speaking, hardwoods are considered as a better case scenario as substrate for enzymatic hydrolysis, because of their lower amount of recalcitrant lignin. Lignin would not only limit enzyme accessibility to cellulose chain, but it also nonspecifically bonds with hydrolytic enzymes, so that makes them have less chances of working on the reaction site on their substrates. Sugar conversion could be consequently influenced by these factors. Schemes 1 through 6 did not yield noteworthy increase in weight loss during hydrolysis of hardwood under 30 mg enzyme charge (Fig. 7).

Schemes 5-6 for hardwood conducted under 15 and 7.5 mg enzyme dosage

The effect of substrate on Schemes 5 and 6 was next assessed on a lower enzyme charge for hardwood green liquor-pretreated pulps (Fig. 8). An enzyme loading of 7.5 mg/g can achieve 56% conversion of green liquor pretreated hardwood pulp for Scheme 6 compared with 25% weight loss as control (the initial cycle which involved no recycling streams).

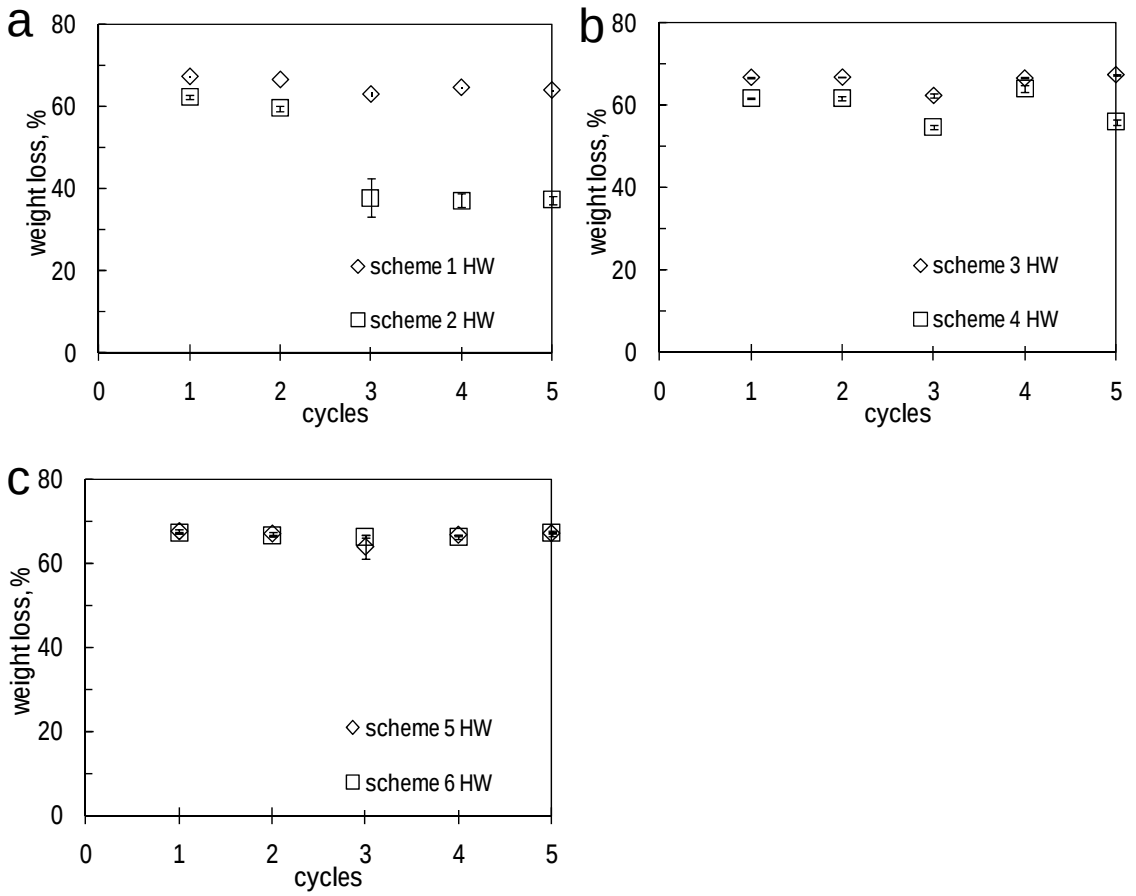

Fig. 7. Weight loss for hardwood from Schemes 1 through 6 at 30 mg enzyme loading per gram of pulp. (**a.** Schemes 1 and 2; **b.** Schemes 3 and 4; **c.** Schemes 5 and 6). Schemes 1, 3, and 5 were performed without surfactant; Schemes 2, 4, and 6 were performed with surfactant.

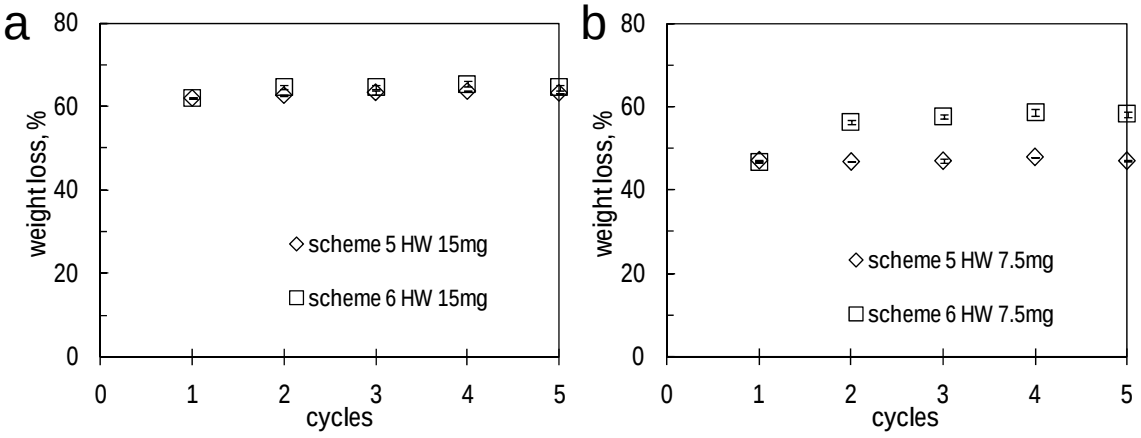

Fig. 8. Weight loss for hardwood with Schemes 5 and 6 at 15 mg (**a.**) and 7.5 mg (**b.**) enzyme loading per gram of pulp. Scheme 5 was performed without surfactant; Scheme 6 was performed with surfactant.

At high enzyme dosages, the benefit from the recycled enzymes is not visible, since the basal sugar yield is too high, and there are enough enzymes in the system. Thus the improvement for the hydrolysis yield is not as marked as for low enzyme loadings. Surfactant also showed positive influence in this recycling method. This result reinforced the finding that the elution step has a positive effect on enzyme recycling. Surfactant can also enhance sugar conversion during recycling processes.

CONCLUSION

In this study six enzyme recycling schemes were evaluated at steady state. The enzyme recycling rate and substrate weight loss at each cycle were monitored as the main parameters of process performance. Green liquor-pretreated softwood and hardwood were both involved in this study to serve as a model of real substrates. From the observations in this project, the conclusive remarks would be:

1. 10% of enzymes were recycled back for the process with use of a washing stage for softwood at 30 mg/g enzyme dosage (Scheme 5), which made hydrolysis efficiency increase by about 20%.
2. Application of surfactant in the process with a washing stage can further elevate softwood hydrolysis to 40% (as in Scheme 6), which is 42% higher than the first cycle.
3. While Scheme 5 brought no improvement in hydrolysis for hardwood at 7.5 mg/g enzyme dosages, Scheme 6 enhanced hydrolysis by 25%.
4. Accumulation of sugar concentration was the main reason for poor performance of a recycling process without a washing stage (Schemes 1 through 4).
5. At the same conditions, hardwood was a better case scenario compared with softwood for enzymatic hydrolysis.

Therefore, the overall recommendation would be to add a washing stage for eluting away sugars from the recycling stream during enzyme recycling process to decrease the the sugar inhibition effect. Moreover, adding 0.1% surfactant could positively affect the recycling process.

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