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One-pot pretreatment, saccharification and ethanol fermentation of lignocellulose based on acid-base mixture pretreatment

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Currently, for the production of cellulosic ethanol, multi-step unit operations, including pretreatment, solid/liquid (S/L) separation, solids washing, liquid detoxification, neutralization, enzymatic hydrolysis and fermentation, are the commonly required steps, which contribute to the elevated capital and operating costs. To simplify these steps, consolidated bioprocessing (CBP), focusing on the multi-functional microbial strains, was proposed but still far from industrialization. In this study, using an acid−base mixture as a pretreatment catalyst, pretreatment, saccharification and fermentation were performed in one pot without S/L separation, neutralization and detoxification. From the one-pot process based on the acid-base mixture pretreatment (190°C, 2 min and 0.15 (w/v) acid−base mixture) and 15 FPU of cellulase/g glucan and *Sacchromyces cerevisiae*, 70.7% of the theoretical maximum ethanol yield (based on the initial amount of glucan in untreated rice straw) was obtained. This was comparable to the estimated ethanol yield (*e.g.* 72.9%) from the assumption of 90% glucan recovery yield after pretreatment × 90% glucose yield from saccharification × 90% ethanol yield from ethanol fermentation, which are performed in three separate pots. These results suggest that whole slurry processing of lignocellulose in one pot could be an attractive way to achieve economic sustainability in the production of fuel from lignocellulose.

Broader context

Production of biofuels using lignocellulosic biomass is attractive due to its sustainability with regards to the environment and energy consumption. However, processing costs in converting lignocellulose into biofuels such as cellulosic ethanol hinders their commercialization. Owing to the rigidity of lignocellulose, the complex multi-step unit operations, including biomass pretreatment, solid/liquid (S/L) separation, solids washing, detoxification or conditioning of liquid, separation of inhibitors, neutralization, enzymatic hydrolysis and fermentation are required. One solution to lower the associated costs is to simplify this multi-step process. In this study, we describe a significantly simplified process, mainly based on pretreatment of lignocellulose using an acid-base mixture that has not been previously exploited as a pretreatment catalyst. Using the acid-rich acid-base mixture as a catalyst enabled "the one-pot pretreatment, saccharification and fermentation" without S/L separation, solids washing, neutralization and liquid detoxification. Using a single pot for the whole process and subsequent conversion of lignocellulose into ethanol will be beneficial in reducing the operating costs involved in cellulosic ethanol production.

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1 Introduction

Due to the environmental benefits and the concern of high dependence on petroleum, the importance of biofuel production from lignocellulose is increasing.[1] The high recalcitrance of lignocellulose needs to be alleviated by using an appropriate physicochemical pretreatment to increase the enzymatic digestibility of cellulose contained in the lignocellulose.[2,3]

Since the physicochemical pretreatment processes are usually performed at extreme pH and/or high temperatures, the generation of sugar degradation products such as 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) are unavoidable.[4-6] These pretreatment byproducts inhibit microorganisms during biofuel synthesis such as ethanol fermentation by yeast.[7]

The process of biofuel production from lignocellulose, which is represented by cellulosic ethanol, involves pretreatment, solid/liquid (S/L) separation, solids washing, liquid detoxification, enzymatic hydrolysis and ethanol fermentation (Fig. 1A). In this process of cellulosic ethanol production, unit operations for the detoxification or removal of inhibitors such as S/L separation, solids washing and liquid detoxification (*e.g.* overliming followed by acidification, chromatographic separation of sugar, etc.) contribute to the significant increase in operating costs.[8-10] To avoid these steps, the development of either inhibitor-tolerable yeast or a novel pretreatment process that does not produce inhibitors is required.

In an attempt to reduce the production cost of cellulosic ethanol, the method of consolidated bioprocessing (CBP) was suggested. This process combines cellulase production, hydrolysis of cellulose and ethanol fermentation in a single step, using a genetically engineered microorganism capable of producing cellulase and fermenting ethanol simultaneously.[11] However, the potential of CBP has not yet been realized.[12] Recently, to simplify the process, "whole slurry fermentation," which involves fermentation of all pretreated lignocellulose slurry without S/L separation, was demonstrated (Fig. 1B).[13-15] Even in this process, a detoxification step such as activated carbon treatment[14] or pH adjustment[15] is needed to remove inhibitors generated during acid pretreatment. In the same context, "one-pot pretreatment and saccharification," which combines ionic liquid (IL) pretreatment and saccharification using an IL-tolerant enzyme cocktail into a single-unit, was presented (Fig. 1C); however, IL needs to be separated and recovered from hydrolysate prior to fermentation. Furthermore, simultaneous saccharification and fermentation (SSF) is not possible in this process configuration.[16]

In this study, we developed an integrated pretreatment, saccharification and fermentation process in one reactor using an acid-base mixture as a pretreatment catalyst. This process does not require S/L separation, neutralization and detoxification either after pretreatment or before SSF (Fig. 1D). This simplified process may highly impact the lignocellulose-based biofuels and biorefinery industries through the substantial reduction in operating costs.

2 Experimental

2.1 Lignocellulose and compositional analyses

Rice straw used in this study was harvested in Yeonggwang, Korea in 2011. Rice straw was washed with tap water, air-dried and milled using a cutting mill (MF 10, IKA; Staufen, Germany). Rice straw was then sieved to generate particle sizes of 90–1000 μm. Carbohydrates and acid-insoluble lignin in rice straw were analyzed following the Laboratory Analytical Procedure (LAP) of the National Renewable Energy Laboratory (NREL; Golden, CO).[17] The sugars, pretreatment by-products and fermentation products in the liquid fraction, as well as the total solids and ash contents, were also measured following the LAP of NREL.[18-20]

2.2 Thermochemical pretreatment of lignocellulose

Ground dry rice straw (2 g) was soaked in 20 mL catalyst solutions comprised of various mixing ratios and concentrations of acid-base mixtures in 100-mL vessels (SK-12 type; Milestone; Shelton, CT) equipped with a thermocouple. Pretreatment was performed by digesting the biomass and catalyst solution mixture in the vessels whilst ramping to 190°C for 3 min and holding at 190°C for 2 min using a microwave digester (ETHOS EZ; Milestone). To prepare samples for the analyses of biomass compositions and enzymatic digestibilities of pretreated biomass, insoluble solids were separated from pretreated slurry by washing with 1 L of distilled water and filtering through a filter cloth (pore size of 22–25 μm; Calbiochem, La Jolla, CA) until the pH of the filtrate reached 6–7. Some of the washed insoluble solids were transferred to aluminum dishes and placed in a vacuum drying oven at 45°C for three days to enable analysis of the solids composition. Other insoluble solids were stored at −20°C for further experiments such as enzymatic hydrolysis and SSF. For whole slurry fermentation of pretreated biomass, the solid and liquid fractions from pretreated slurry were directly proceeded to SSF without S/L separation. To quantify sugar monomers, including glucose, xylose, galactose, arabinose and mannose and to quantify ethanol and other byproducts, including furfural, HMF, acetic acid, formic acid and levulinic acid, Aminex HPX-87P (Bio-Rad; Hercules, CA) and Aminex HPX-87H (Bio-Rad) columns were used, respectively, for high pressure liquid chromatography (HPLC; Agilent Technologies, Waldbronn, Germany) as previously described.[14,15]

2.3 Enzymatic hydrolysis of lignocellulose

To evaluate the effectiveness of the pretreatment, untreated rice straw or pretreated and washed rice straw was enzymatically hydrolyzed using 15 FPU/g glucan of a commercial cellulase (Accellerase 1000; Genencor, Rochester, NY), following the LAP of NREL.[21] In brief, lignocellulosic biomass with 1% (w/v) of the final glucan concentration was added to 10 mL of 0.05 M sodium citrate buffer (pH 4.8) at 50°C. The enzymatic digestibility was expressed as the percentage of the theoretical maximum glucose produced per the total amount of input glucose. HPLC equipped with an Aminex HPX-87P column was used to measure the amount of glucose from the enzymatic hydrolysis.

2.4 Simultaneous saccharification and fermentation

SSF was carried out to produce ethanol from untreated or pretreated rice straw following the LAP of NREL with a slight modification.[22] Comparable to the protocol followed for glucan and biomass loadings, the final glucan concentration of 3% (w/v) for untreated or pretreated and washed rice straw, and the final biomass concentration of 6% (w/v) based on untreated rice straw for the whole slurry fermentation, were used in the SSF. After autoclaving the SSF media (1% yeast extract, 2% peptone and 0.05 M citrate buffer at pH 4.8) at 121°C for 20 min, 15 or 60 FPU of Accellerase 1000/g glucan and 5% (v/v) of *Saccharomyces cerevisiae* D_5A (ATCC 200062)—grown in YPD media containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose—were added. Particularly for the whole slurry fermentation of the acid-base pretreated rice straw, the SSF media components were added into the pretreated slurry after the acid-base pretreatment without any operation. SSF was conducted in a flask with a needle-pierced silicone stopper to vent the CO_2 produced during fermentation, in a shaking incubator for 60 h at 38°C and 170 rpm. Ethanol yields were determined as the percentage of the theoretical maximum based on the glucan contained in the rice straw before pretreatment.

2.5 Evaluation of cellulose accessibility to the enzyme

To analyze the cellulose accessibility of pretreated rice straw to cellulase, the binding capacity of rice straw to a typical carbohydrate-binding module (CBM) of cellulase was quantified using a Type A surface binding CBM from *Clostridium thermocellum* (*Ct*CBD3).[23-26] Recombinant *Ct*CBD3 was prepared as previously described.[25,26] Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) was used as the control protein in binding experiments. For the binding analysis, 5 mg of substrates (untreated or pretreated rice straw) were incubated with an excess amount (0.4 nM) of BSA or *Ct*CBD3 in 500 μL of 0.05 M potassium phosphate buffer (pH 7). After 3.5 h, the binding mixture was separated into unbound protein in the supernatant, and bound protein in the pellet by centrifugation at 25,000 × *g* for 5 min. The amount of unbound protein was measured by the Bradford method.[27] The amount of bound protein was determined by subtracting the amount of unbound from the total protein initially added to the binding mixture.

3 Results and discussion

3.1 Effects of acid-base concentrations and mixture ratios on pretreatment

Previous studies have suggested pretreating lignocellulose by sequential applications of acid and base[28-30] or by using salt as a catalyst.[31-33] However, to the best of our knowledge, using a mixture of acid and base together has not been attempted for pretreating lignocellulose. When different mixing ratios of acid and base (*i.e.* HCl and NaOH of 1:4 to 4:1) were tested, the acidic region of the acid-base mixture gave the highest glucose yield (Fig. 2A). Other ratios of acid-base mixtures representing neutral or alkaline pH gave glucose yields lower than 15 g per 100 g of lignocellulose. The acid-base molar ratio of 4:1 was selected for further pretreatment experiments.

Based on the optimal acid-base mixture ratio selected as shown in Fig. 2A, various total concentrations of the acid-base mixture (0.01–1 M) were tested for the pretreatment of rice straw at 190°C (Fig. 2B). In the case of pretreatment without any acid-base mixture, the enzymatic digestibility was higher than that of untreated rice straw, probably due to the slight removal of lignin by the acidic nature of water at high temperature.[34] As the total concentrations of acid-base mixtures increased from 0 M to 0.05 M, the glucose yield significantly increased (from 9.6 g to 27.2 g per 100 g of lignocellulose). However, a further increase in the concentration of the acid-base mixture did not result in any significant increases in enzymatic digestibility. For example, when the total concentrations of the acid-base mixtures increased to 0.5 M or 1 M, the produced glucose yield was significantly decreased since the recovery yield of insoluble solids was reduced to lower than 40%. Therefore, 0.05 M acid-base mixture (0.04 M of HCl and 0.01 M of NaOH) was selected as the optimum concentration for the pretreatment of rice straw, in which the enzymatic digestibility of 75.9% of the theoretical maximum glucose was obtained using pretreated and washed rice straw. Although the optimum molar concentration of acid-base mixture used in this study, which is equivalent to 0.15% (w/v) of HCl and 0.04% (w/v) of NaOH, is much lower than those of acid (0.5–5% [w/v])[35,36] or base (0.5–3% [w/v] for NaOH[37] and 2–14% [w/w] for NH_3)[38,39] catalysts in other pretreatment studies, this enzymatic digestibility is comparable to those obtained from the conventionally pretreated rice straw using acid or base, which showed ~70% yields.[12,39,40]

3.2 Effects of acid, base and salt on pretreatment

To investigate the mechanism of pretreatment using the acid-base mixture, several control experiments were performed using combinations of catalysts such as acid (HCl), base (NaOH), salt (NaCl) and acid with salt (Fig. 3). In these experiments, the amount of salt (*i.e.* 0.01 M NaOH) added to the acid was determined using the molar ratio in the acid-base mixture (*i.e.* 0.04 M HCl and 0.01 M NaOH) to simulate the formation of salt in the acid-base mixture. Among the different combinations of acid, base and salt, the acid-base mixture gave the highest enzymatic digestibility to the pretreated and washed rice straw (Fig. 3). The HCl pretreatment resulted in an enzymatic digestibility of 68.8% of the theoretical maximum glucose yield, but NaOH or NaCl resulted in 32.0% and 33.8% enzymatic digestibilities, respectively. In particular, the combination of HCl and NaCl, which was designed to simulate the acid-base mixture involving the acid-base reaction, did not result in enzymatic digestibility as high as that found with the acid-base mixture. Therefore, the possible effect of the formation of NaCl and the remaining HCl from the acid-base mixture in the rice straw pretreatment was not simulated by the mixture of NaCl and HCl.

The compositions of rice straw pretreated using the acid-base mixture, HCl, NaOH or NaCl were analyzed (Table 1). In the untreated rice straw, the total amount of carbohydrate and lignin was 70.9%, and this value was comparable to the amount derived from rice straw in other studies.[39,40] The recovery yields of insoluble solids following NaOH or NaCl pretreatment were much higher than those following the acid-base mixture, or HCl pretreatment. This can be related to the lower enzymatic digestibility following NaOH or NaCl pretreatment compared with the acid-base mixture or HCl pretreatment. In particular, xylan was substantially reduced after pretreatment when using HCl or the acid-base mixture. Glucan was also reduced, but to a lesser degree. These results are consistent with the typical characteristics of acid pretreatment.[14,41] Specifically, in the acid-base mixture pretreatment, the recovery yields of glucan, xylan and lignin in the insoluble solids were 90.1, 37.2 and 60.6%, respectively. Accordingly, a higher amount of xylose was recovered in the liquid fraction of pretreated rice straw when using the acid-base mixture or HCl, compared to when using NaOH or NaCl. When using the acid-base mixture, the lignin removal was comparable with that when using NaOH; however, the generation of acetic acid was lower than that when using HCl. Also, when using the acid-base mixture, furfural and HMF productions were lower than when using HCl. All of these results indicate that the acid-base mixture pretreatment is advantageous due to the formation of fewer inhibitors than are produced following acid or alkali pretreatment. Therefore, the acid–base mixture pretreatment has the positive aspects of acid and alkali pretreatments, such as solubilizing hemicellulose and removing lignin, respectively.

3.3 Correlation of xylan and lignin removal with the enzymatic digestibility of acid-base mixture pretreated rice straw

The amounts of major biomass components of pretreated rice straw, such as glucan, xylan and lignin, were correlated with the enzymatic digestibilities of the acid-base mixture pretreated rice straw (Fig. 4). The removal of glucan did not show a high correlation coefficient with an increase in enzymatic digestibility. However, the losses of both xylan and lignin after the acid-base mixture pretreatment were highly correlated with an increase in enzymatic digestibility. These results imply that the removal of hemicellulose (*e.g.* xylan) and lignin may have contributed to the increased enzymatic digestibility by the pretreatment using the acid-base mixture. It is well known that removal of hemicellulose contributes to the increase of enzymatic digestibility in acid pretreatment[41] and that the removal of lignin contributes to the increase of enzymatic digestibility in alkali pretreatment.[38,42] The acid-base mixture pretreatment used in this study, resulted in the significant removal of both hemicellulose and lignin, which is known to increase enzymatic digestibility of pretreated biomass.

3.4 Evaluation of cellulose accessibility to the enzyme using a CBM

The cellulose accessibility of the acid–base mixture pretreated rice straw to enzyme was tested by protein binding analysis using *Ct*CBD3, a Type A CBM that is the typical CBM for cellulose surface binding in cellulase.[24] Untreated and pretreated rice straw samples did not show significant differences in non-specific protein binding using BSA as a control (Fig. 5). When *Ct*CBD3 was used, the pretreated rice straw exhibited approximately two times higher binding capacity than untreated rice straw. These results suggest that the acid–base mixture pretreatment significantly improved cellulose accessibility to the CBM, which may have contributed to the increased enzymatic digestibility of the acid-base mixture pretreated rice straw as shown in Fig. 3. In a previous study using alkali pretreatment of lignocellulose with more than 40% lignin removal,[42] *Ct*CBD3 binding was not higher than in untreated lignocellulose. This was probably due to the redistribution or condensation of lignin after alkali pretreatment.[42,43] However, despite a similar degree of lignin removal in this study, removal of both lignin and hemicellulose may have transformed the pretreated rice straw into a structure that was more accessible to the CBM.[26,44] Moreover, transformation of lignin structures by acid in the acid-base mixture may reduce unproductive binding of CBM to lignin.[45] This unique feature of the acid–base mixture pretreatment in the removal and modification of both hemicellulose and lignin, improves the accessibility of cellulose in lignocellulose to enzyme.

3.5 Saccharification and ethanol fermentation of the acid-base mixture pretreated rice straw

Washed solids of rice straw pretreated at the optimal conditions (0.05 M acid-base mixture composed of 0.04 M HCl and 0.01 M NaOH and 3 min ramping to 190°C and 2 min holding at 190°C) were hydrolyzed with 15 FPU Accellerase 1000/g glucan (Fig. 6A). The glucose yield from untreated rice straw (control) was only 15.6% of theoretical maximum glucose at 72 h. The final glucose yield at 72 h from the acid-base mixture pretreated and washed rice straw was 75.2%; however, more than 70% of the final glucose yield was achieved after 6 h. This relatively fast hydrolysis is uncommon in pretreated lignocellulose since other studies have reported that approximately 40–65% of glucose yields are obtained after 6–12 h.[14,38,39] Depending on the process economics, it would be preferable to compromise the hydrolysis time rather than a lower maximum glucose yield. The fast reactivity of the acid–base pretreated rice straw (Fig. 6A) can be attributed to the high cellulose accessibility to the enzyme (Fig. 5).

To test the applicability of the acid-base mixture pretreatment in "the one-pot pretreatment, saccharification and fermentation," rice straw was pretreated with the acid-base mixture and then directly proceeded to the SSF by addition of 15 FPU Accellerase 1000/g glucan with buffer (0.05 M) and *S. cerevisiae* D_5A, in which neutralization, conditioning, or detoxification steps were not performed (Fig. 6B). After acid pretreatment of lignocellulose, to utilize the entire liquid faction

or the whole slurry of pretreated biomass, conditioning or neutralization of the liquid fraction is required.[14,46] Even after neutralization of acid, the formation of salts has been shown to inhibit microbial cell growth.[14,15] However, in this study neutralization and conditioning were not necessary. It is likely that this was due to the concentration of acid-base mixture being less than 10 times lower than conventional dilute-acid pretreatments, as well as the partial neutralization in the acid-base mixture. Furthermore, an inhibitory effect of salts could have been negligible due to the low amount of acid and base used in the pretreatment during this study. Although the ethanol yield was determined based on the amount of glucan before pretreatment, in which all the losses of glucan during pretreatment were accounted for, the ethanol yield from the whole slurry fermentation after 60 h reached 70.7% with 15 FPU of enzyme. This ethanol yield was comparable to the ethanol yield (*e.g.* 72.9%) estimated based on the assumption of 90% glucan recovery yield after pretreatment × 90% glucose yield from saccharification × 90% ethanol yield from ethanol fermentation. When only the washed solids of the pretreated rice straw were used in the SSF, the maximum yield of ethanol was only 49.7% with 15 FPU of enzyme (after 48 h) based on the initial glucan before pretreatment. The ~30% lower ethanol yield when using the washed solids only compared with the fermentation of the whole slurry, was due to the liquid fraction of the pretreated slurry being discarded. Similarly, a previous study with using dilute-acid pretreated and washed corn stover, only 41.9% ethanol yield based on both glucan and xylan amounts before pretreatment was obtained because of the sugar loss during S/L separation and solids washing.[47] Therefore, whole slurry fermentation has the advantage of using all the available glucan and glucose both in the solid and liquid phases of the pretreated slurry.[14,15,48] Moreover, the acid-base mixture pretreatment has the advantage of both enhancing final ethanol yield, and eliminating the conditioning and neutralization step.

3.6 Generalization of acid-base mixture pretreatment

To validate the pretreatment effectiveness of the acid-base mixture as a pretreatment catalyst, different combinations of acid-base mixtures other than the HCl-NaOH mixture were tested using H_2SO_4, KOH and NH_3. In these experiments, pretreatment was performed using the acid-base molar ratios of 4:1, 1:1 and 1:4 in the acid-base mixture, but other pretreatment conditions were fixed at the optimal pretreatment conditions selected for the HCl-NaOH mixture (Supplementary Fig. 1). Of all the acid-base combinations, the acid-rich acid-base mixture (*i.e.* the molar ratio of 4:1 = acid: base) resulted in significantly higher enzymatic digestibility of pretreated and washed rice straw than equal or base-rich acid-base mixtures. For example, when H_2SO_4–KOH (Supplementary Fig. 1B) was used, the highest enzymatic digestibility, 73.9%, was obtained. H_2SO_4–NaOH (Supplementary Fig. 1A) and H_2SO–NH_3 (Supplementary Fig. 1C) mixtures resulted in ~65% enzymatic digestibility. HCl–KOH (Supplementary Fig. 1D) and HCl–NH_3 (Supplementary Fig. 1E) mixtures resulted in approximately 50–60% enzymatic digestibilities. Generally in lignocellulose pretreatment, removal of hemicellulose by acid gives more positive effects on enhancing enzymatic digestibility than removal of lignin removal by alkali.[49] These results indicate that an acid-rich acid-base mixture may be used as an effective catalyst through optimizing total concentration and other pretreatment conditions.

3.7 Process overview

Based on the results of this study, 100 g dry wt of rice straw is pretreated with the acid-base mixture and is added to 0.05 M sodium citrate buffer, 15 FPU cellulase/g glucan and *S. cerevisiae* for the SSF in the same reactor that was used for the pretreatment (Fig. 7). From this one-pot pretreatment, saccharification and fermentation, 14.4 g ethanol is obtained, which is 70.7% of the theoretical maximum ethanol from initial glucan in rice straw before pretreatment, considering glucan loss throughout the process. When the insoluble solids obtained from washing the pretreated rice straw were used for the SSF, only 10.1 g of ethanol (49.7% of the theoretical maximum) is obtained since the liquid phase of the pretreated rice straw containing residual sugars and inhibitors is not used in the fermentation. Since high titer of ethanol production is also important to achieve economic sustainability in the production of fuels and commodity products from lignocellulose,[48] the proposed one-pot pretreatment, saccharification and fermentation should be investigated at high solids loadings of lignocellulose in the future. Furthermore, due to the large amount of xylose solubilization during the pretreatment in this study, if a yeast engineered to ferment xylose is used in ethanol fermentation, overall ethanol production could be significantly increased.

3.8 Comparison of estimated ethanol production costs

Although pretreatment, saccharification and fermentation in one pot may provide advantages in the process economics, but many other factors such as enzyme cost, reaction conditions used, saccharification and fermentation efficiency etc. are also important in determining the commercial success of fuel production using lignocellulosic biomass. Therefore, using the most updated NREL's cellulosic ethanol production cost estimation of different process schemes, cost analysis of the one-pot process of the present study was implemented and compared.[8] The four scenarios are mainly differentiated by the mode of operation, which are separate conditioning and separate fermentation (SCSF), separate conditioning and whole slurry fermentation (SCWF), whole slurry conditioning and fermentation (WCF) and acid-base mixture one-pot pretreatment, saccharification and fermentation (ABM one-pot; this study) (Supplementary Fig. 2). SCSF is very conventional and already proven technology, and SCWF is also conventional but is on-going technology. WCF is one of the advanced technologies but is not proven yet, which will be available soon. However, the process models of SCSF, SCWF and WCF are

not accurately representing an existing industrial process since no commercial cellulosic ethanol plants exist to date. The most advanced model technology (*i.e.* WCF) and ABM one-pot have high similarity such as less consumption of catalysts, neutralizing agents and enzyme; less time duration for saccharification and fermentation; and slightly lower ethanol yields due to less enzyme dosage.

On the basis of the scenarios, estimated costs for the production of a gallon of ethanol were compared, considering operating costs and installed equipment costs (Fig. 8). The costs of ethanol for SCSF, SCWF, WCF and ABM one-pot were \$6.47, \$6.42, \$5.95 and \$5.07, respectively. A detailed contribution of each factor was presented by Supplementary Table 1. Compared to WCF, ABM one-pot showed lower ethanol production costs due to the less loading of catalysts, no need of neutralizing agents and water washing, and lower non-enzyme conversion-related costs. In addition, less loading of enzyme leads to a further decrease in enzyme conversion-related cost. Since S/L separation, solids washing, conditioning and pH adjustment using neutralizing agents are not necessary in the ABM one-pot process, relevant equipment costs were deducted. Although the ethanol yield of this study, 70.7 gallons/dry ton of rice straw, was lower than that of NREL study (*i.e.* 79 gallons/dry ton of corn stover), the ABM one-pot process reduced other facility costs such as boiler, storage, etc. Taken together, by estimating the ethanol production costs, it was identified that the ABM one-pot pretreatment, saccharification and fermentation scheme would be economically promising. However, to increase ethanol yields and titers, further work regarding high loadings of solids needs to be investigated.

4 Conclusions

The majority of current lignocellulosic pretreatments require S/L separation, washing or neutralization and even detoxification after pretreatment and before SSF or separate hydrolysis and fermentation (SHF), which generate additional costs. Therefore, to establish a cost-effective cellulosic ethanol process, post-pretreatment steps need to be minimized. The "whole slurry processing in one pot" described here may provide a solution. Therefore, the pretreatment should not use too much acid or base catalysts so as not to generate large amounts of inhibitors. Considering all the costs with regards to the post-pretreatment processing steps in the conventional chemical pretreatment, the one-pot process, integrating the pretreatment, saccharification and fermentation, which is based on the acid-base pretreatment, gives apparent advantages in the process economics for producing a commodity product using lignocellulose, although the exact mechanism of the acid-base pretreatment has not been revealed.

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Figure titles

Fig. 1 Schematic diagrams showing the pretreatment, saccharification and fermentation processes for cellulosic ethanol production. (A) conventional multi-unit configuration, (B) whole slurry fermentation configuration,[14] (C) one-pot pretreatment and saccharification configuration[16] and (D) the novel "one-pot pretreatment, saccharification and fermentation processes."

Fig. 2 Effects of (A) different molar ratios of acid (HCl)-base (NaOH) mixtures (*i.e.* total concentrations of 0.05 M) and (B) total concentrations of acid-base mixtures with the acid-base molar ratio of 4:1 on the enzymatic digestibility of pretreated and washed rice straw on the basis of total dry wt of input untreated rice straw. Pretreatment was conducted using

various concentrations of acid-base mixture at 190°C and a solids loading of 10% (w/v) with 3 min ramping to 190°C and 2 min holding at 190°C in a microwave digester. Enzymatic hydrolysis was conducted using 15 FPU Accellerase 1000/g glucan at pH 4.8, 50°C and 200 rpm for 50 h.

Fig. 3 Enzymatic digestibilities (% theoretical maximum glucose from remaining glucan in pretreated and washed rice straw) of rice straw samples pretreated with various catalysts: 0.04 M HCl; 0.01 M NaOH; an acid-base mixture of 0.04 M HCl and 0.01 M NaOH; 0.01 M NaCl; 0.01 M NaCl; and 0.03 M HCl. Enzymatic hydrolysis was conducted using 15 FPU Accellerase 1000/g glucan at pH 4.8, 50°C and 200 rpm for 50 h.

Fig. 4 Correlation of the biomass components of rice straw pretreated using the acid-base mixtures under various conditions (*e.g.* pHs and total concentrations of acid-base mixtures) with the enzymatic digestibilities. (A) glucan, (B) xylan and (C) lignin.

Fig. 5 Comparison of the protein binding capacities of untreated rice straw and the acid–base mixture pretreated rice straw. Rice straw was incubated with BSA or *Ct*CBD3 in 50 mM potassium phosphate buffer (pH 7) at 4°C for 3.5 h.

Fig. 6 (A) Saccharification of washed solids of the acid–base mixture pretreated rice straw using 15 FPU of Accellerase 1000/g glucan at pH 4.8, 50°C and 200 rpm. (B) Simultaneous saccharification and ethanol fermentation (SSF) of the whole slurry or the washed solids of the acid–base mixture pretreated rice straw. For the SSF, 15 FPU Accellerase 1000/g glucan and *Saccharomyces cerevisiae* D_5A were added and cultivation was performed at pH 4.8, 50°C and 170 rpm for 60 h.

Fig. 7 Mass balances for the processing of rice straw to produce ethanol. Rice straw was pretreated with the HCl (0.04 M)-NaOH (0.01 M) mixture at 190°C. Pretreated rice straw was hydrolyzed with 15 FPU of Accellerase 1000/g glucan and fermented with *S. cerevisiae* D_5A at 38°C for 60 h. The ethanol yield was determined based on the total glucan contained in untreated rice straw before pretreatment.

Fig. 8 Comparison of estimated ethanol production costs for four different process schemes such as separate conditioning and separate fermentation (SCSF), separate conditioning and whole slurry fermentation (SCWF), whole slurry conditioning and fermentation (WCF) and one-pot pretreatment, saccharification and fermentation using acid-base mixture (ABM one-pot). Costs were estimated on the basis of NREL Technical Report.[8]

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Table 1. Composition of rice straw pretreated with different catalysts

	Untreated	Acid-base mixture (0.04 M HCl-0.01 M NaOH)	HCl (0.04 M)	NaOH (0.01 M)	NaCl (0.01 M)
Component from insoluble solids (g per 100 g dry rice straws before pretreatment)					
Insoluble solids recovery yield	NA[d]	55.6 ± 2.5	55.9 ± 2.6	75.5 ± 0.8	85.0 ± 3.9
Glucan	35.8 ± 1.5	32.3 ± 0.6	30.9 ± 0.3	33.7 ± 0.0	33.1 ± 0.4
Xylan	10.5 ± 1.4	3.9 ± 0.5	2.9 ± 0.2	10.7 ± 0.2	10.5 ± 0.4
Galactan	3.3 ± 0.3	1.5 ± 1.4	2.2 ± 0.0	2.9 ± 0.0	3.4 ± 0.0
Arabinan	3.1 ± 0.5	1.8 ± 0.0	1.7 ± 0.0	2.3 ± 0.0	2.6 ± 0.0
Lignin	18.2 ± 1.3	11.0 ± 0.3	10.7 ± 0.0	10.8 ± 0.3	12.2 ± 0.2
Component from dissolved solids (g per 100 g rice straw before pretreatment)					
Glucose	NA[d]	4.9 ± 0.2	6.1 ± 0.0	0.5 ± 0.0	1.5 ± 0.0
Hemicellulosic monomer[c]	NA[d]	10.2 ± 0.1	14.9 ± 0.1	0.5 ± 0.0	1.3 ± 0.1
Acetic acid	NA[d]	1.5 ± 0.0	2.0 ± 0.1	1.6 ± 0.0	0.3 ± 0.1
HMF	NA[d]	0.4 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Furfural	NA[d]	0.6 ± 0.0	0.7 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Pretreatment conditions were 190°C, 3 min ramping with 2 min holding time and 10% (w/v) solids loading

[b] Experimental data are expressed as means ± standard deviations

[c] Includes xylose, galactose and arabinose in liquid fractions

[d] NA: not applicable

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Fig. 1

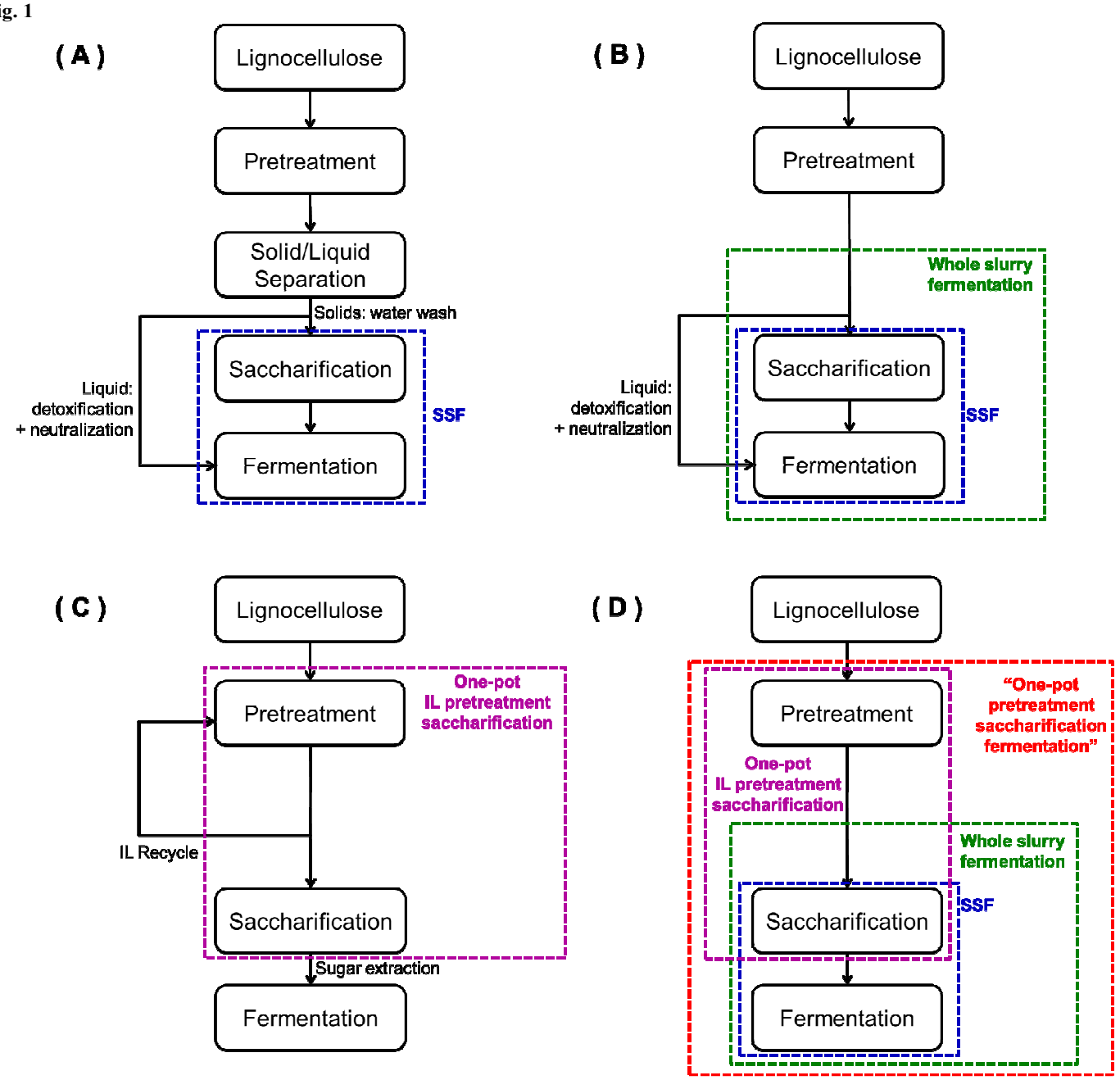

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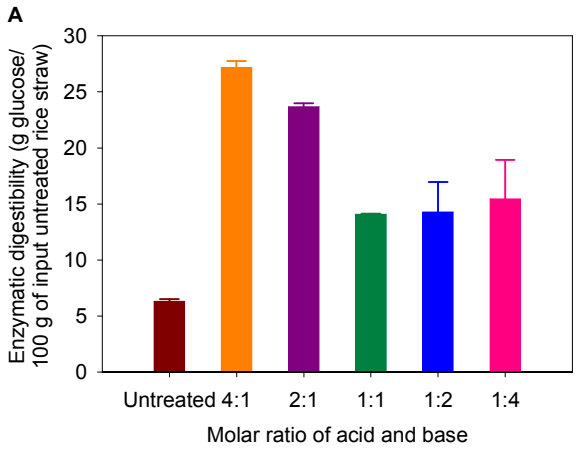
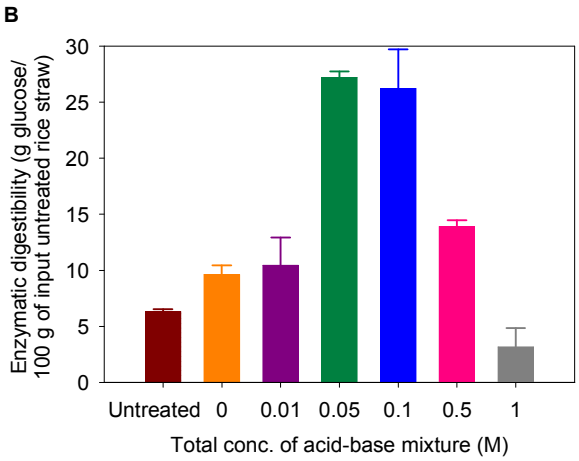
Fig. 2





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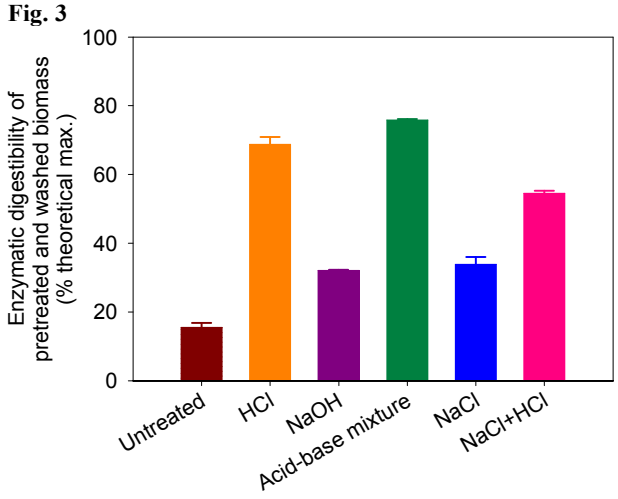

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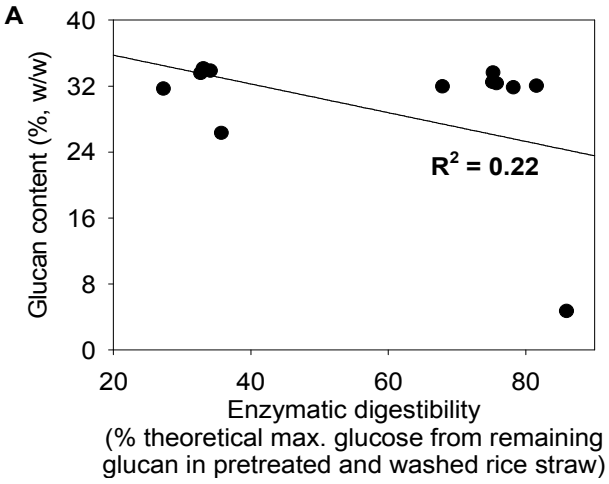

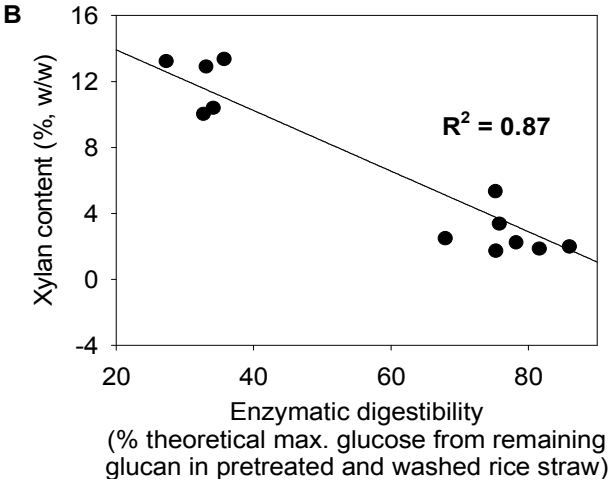

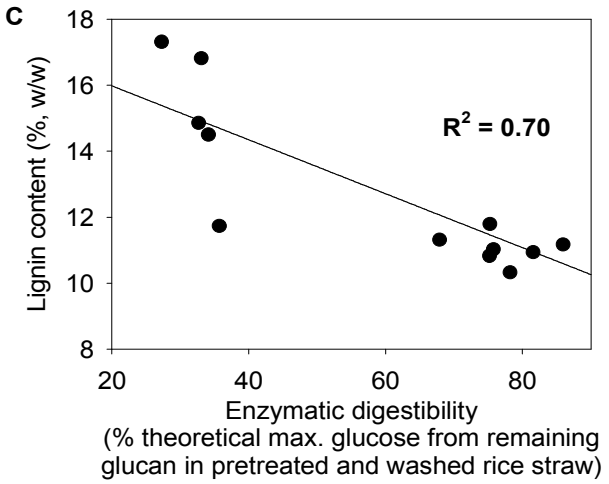

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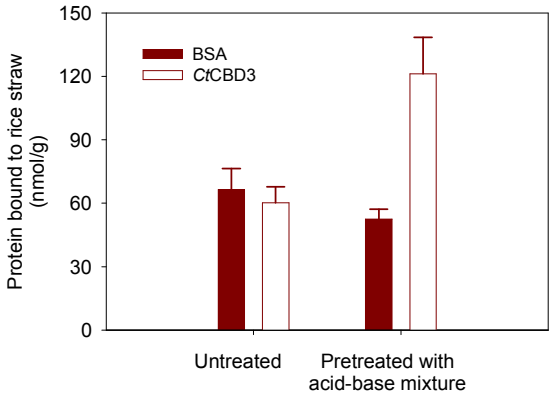

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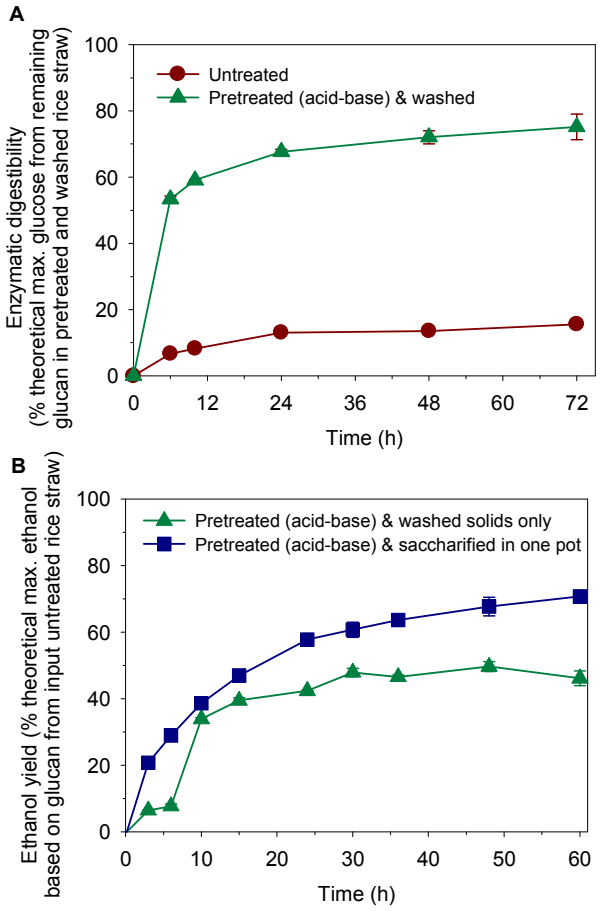

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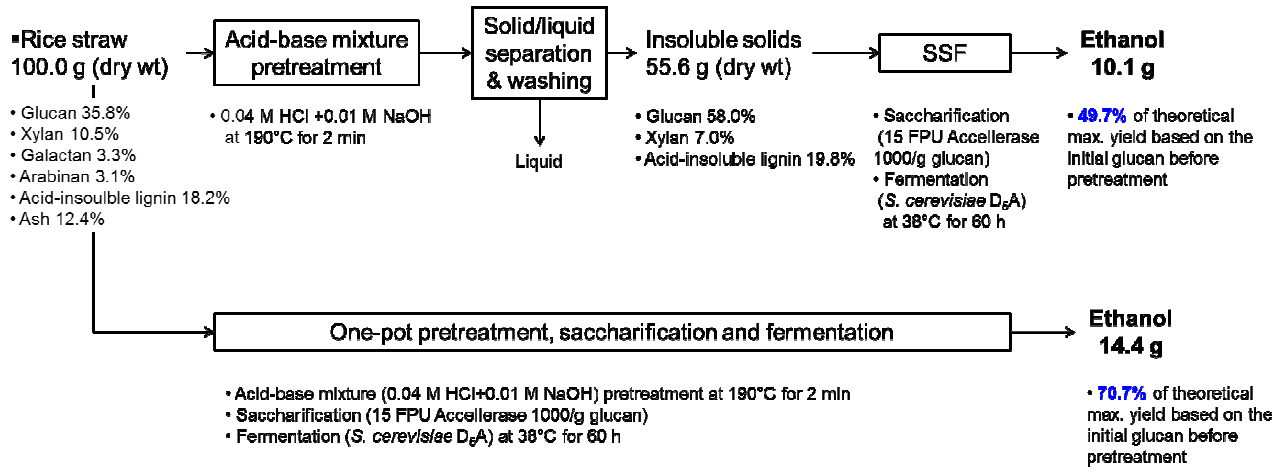
Fig. 7



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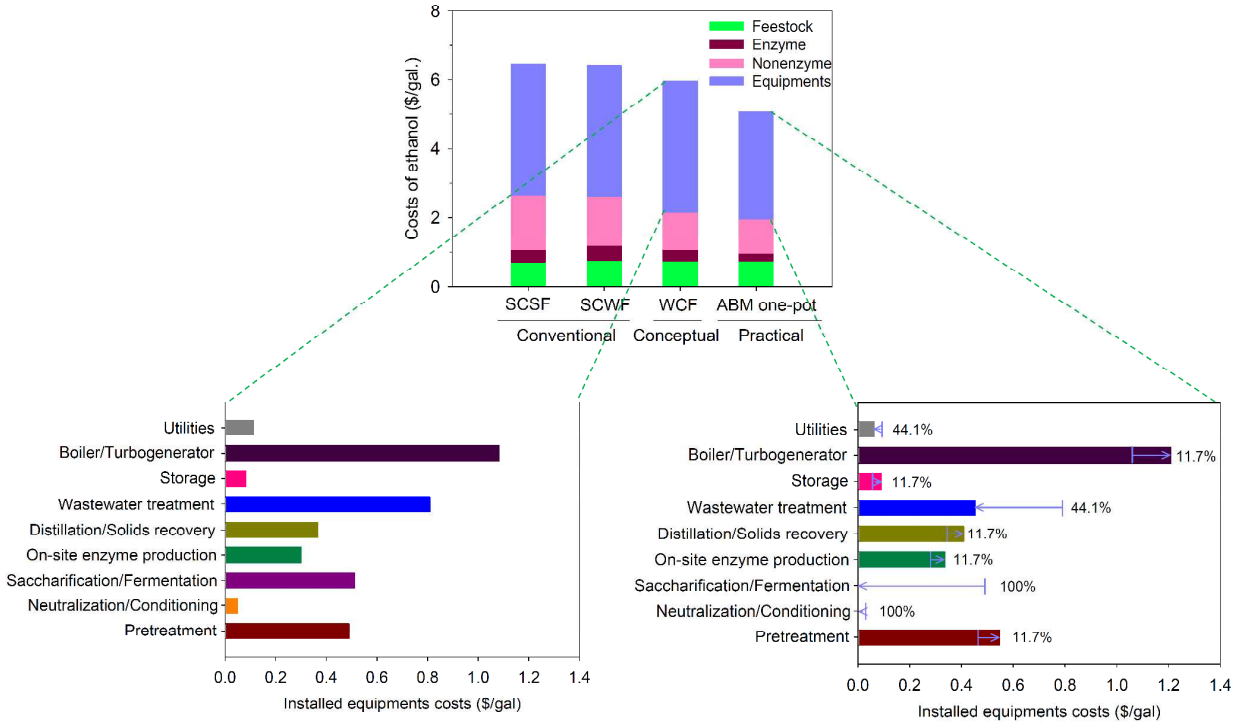

Graphical abstract

One-pot pretreatment, saccharification and ethanol fermentation of lignocellulose, which was established based on acid-base mixture pretreament, will greatly reduce overall processing costs not only for the production of cellulosic ethanol but also for the lignocellulose-based biorefinery. This is because the multi-step unit operations, including solid/liquid separation, detoxification of inhibitors and neutralization of hydrolysates will not be required for the proceessing and conversion of lignocellulosic biomass into fuels and chemicals.