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# Improving alkaline pretreatment method for preparation of whole rice waste biomass feedstock and bioethanol production

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**Abstract**

Efficient conversion of fermentable sugars from cheap lignocellulosic biomass is a current need for viable ethanol production technology. This study investigates the efficiency of individual and multiple chemical pretreatment for the saccharification of whole rice waste biomass (RWB). Individual alkaline pretreatment combined with autoclaving provided a maximum yield of 505 mg of reducing sugar with 69.2% hydrolysis yield after two-step enzymatic hydrolysis using 20 filter paperase (FPU) $g^{-1}$ of RWB. Sequential pretreatment of acidified sodium chlorite and sodium bicarbonate to alkaline pretreated RWB resulted in maximum yield of 725 mg of reducing sugar with hydrolysis yield of 90.6% using a lower enzyme dose (10 FPU $g^{-1}$ of RWB), indicating the feasibility of the process. The effects of different pretreatments on chemical composition and structural complexity of whole RWB and its relation with saccharification were investigated using various analytical techniques. Finally, the enzymatic hydrolysates of pretreated RWB were found suitable for ethanol production. The maximum sugar consumption and ethanol yield by *Saccharomyces cerevisiae* SR8 were 95% and 0.465 g $g^{-1}$ of sugar, respectively. The developed sequential pretreatment showed significant delignification and saccharification of whole RWB at low enzyme dosage. Also efficient conversion of the hydrolysate to ethanol without detoxification demonstrated the feasibility of the process.

Keywords: Rice waste biomass, alkaline pretreatment, acidified sodium chlorite (ASC) pretreatment, enzymatic hydrolysis, *Saccharomyces cerevisiae* SR8

## 1. Introduction

Continuous depletion of existing fossil fuel reserves, and the associated price and concern in global climate changes have prompted scientists to focus attention on the production of sustainable and renewable alternative energy sources such as biofuels.[1,2] As the demand for feedstock for biofuel intensifies, a conflict between food and energy resources has emerged as a critical issue.[3] Development of biofuel production from abundant lignocellulosic biomass such as agricultural and forest residual waste is a practical, cost effective, and a sustainable solution to the energy crisis.[4] Lignocellulosic material is composed of highly crystalline cellulose and hemicellulose polymers where lignin acts as an encrusting agent making the structure more recalcitrant to hydrolysis.[5] To initiate ethanol production from lignocellulosic biomass, a pretreatment step is required for its conversion into fermentable sugars.[6] The selection of pretreatment mainly depends on its cost, delignification efficiency, effectiveness to decrease the crystallinity of cellulose allowing high enzyme accessibility, and presence of the generated inhibitors having adverse effects on hydrolytic enzymes and fermentative microorganisms.[7]

Although a range of physical, chemical, and biological processes have been configured to release sugars from lignocellulose, technical challenges remain for the commercialization of the process.[8,9] Acid pretreatment of lignocelluloses was found to be a very effective and well-known process for acquiring a suitable structure for enzymatic hydrolysis. However, at high temperature and pressure, this process produces certain inhibitory compounds that have negative impact on the downstream process.[5,10,11] Alkaline pretreatment is more efficient to cleave hydrolysable linkages in lignin and glycosidic bonds of polysaccharides, as well as disruption of the lignin structure. As such, it was found to be more effective for biomass, having lower lignin content similar to herbaceous crops and agricultural residues.[7,12] In contrast to acid and alkali treatments, sodium chlorite acidified by

acetic acid (ASC) is a powerful oxidizing agent that meets the requirement for delignification with reduced solubilization of glucan and xylan.[13,14] Moreover, sodium bicarbonate was shown to be useful for swelling the macro- and micro-fibrils of the biomass. Swelling after delignification is necessary to enhance the enzyme accessibility of cellulose for maximum recovery of sugars.[15]

Enzymatic hydrolysis of lignocellulosic biomass into fermentable sugars is considered the most efficient process for saccharification. However, it requires large quantities of cellulolytic enzymes, which makes the process more expensive.[16] To overcome these obstacles, it is necessary to develop an efficient pretreatment technology that can remove lignin and disintegrate microfibrils, and one that also utilizes a low dose of enzyme for improved saccharification.[6,8] In order to achieve these goals, we investigated the effects of different pretreatment strategies, as well as the effects of acidified sodium chlorite and swelling by sodium bicarbonate in combination with alkaline pretreatment for significant delignification and better saccharification of rice waste biomass (RWB) using a low enzyme dose. After enzymatic hydrolysis of biomass, a mixture of C6 and C5 soluble sugars are typically generated. To accomplish higher and cost effective ethanol production, the fermentative microorganism used should have the ability to ferment all sugars (both C6 and C5 sugars) derived from cellulosic biomass, and should also have tolerance to inhibitors formed during the pretreatment.[17] *Saccharomyces cerevisiae* does not natively assimilate xylose (the second most abundant sugar in lignocellulose) due to the lack of xylose-metabolizing enzymes.[18] Therefore, we utilized an enhanced *S. cerevisiae* strain that, due to metabolic engineering, possess the ability to efficiently metabolize both glucose and xylose into ethanol.[17]

It is estimated that in South Korea, about 1.3 million tons of RWB is produced annually from the 684,000 hectares of rice fields.[4] RWB generated after seed harvesting is a

particularly attractive lignocellulosic material for alternative biofuel production.[19] In this study, we explored the potential of different pretreatment options on the digestibility of whole RWB and also determined its hydrolysis yield. The effects of individual and multiple pretreatments in combination with alkaline pretreatment was evaluated in terms of various aspects such as structure and chemical composition of biomass, lignin removal, microscopic morphology, crystallinity, and its saccharification using commercially available cellulase. Finally, the resulting RWB hydrolysates were utilized for fermentative ethanol production by a metabolically engineered *Saccharomyces cerevisiae* SR8. The aim of this work was to evaluate the feasibility of using multiple effects of pretreatment on the hydrolysis yield of prepared biomass feedstock in the enzymatic step as well as for ethanol production.

## 2. Materials and methods

### 2.1. Biomass collection and preparation

Rice (*Oryza sativa*) plant biomass was collected from a local farm in South Korea. Whole plant body, except the roots, was used as the substrate for pretreatment studies. Biomass was chopped, washed with water, and then dried at 60°C. It was then milled, subsequently sieved through 0.2 mm screens, and stored at 4°C until use. Commercial *Trichoderma reesei* cellulase (Celluclast 1.5 L) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used for the pretreatment, enzymatic hydrolysis, and ethanol fermentation experiments were of the highest purity available and of analytical grade.

### 2.2. Chemical pretreatment methods for rice waste biomass (RWB)

During the chemical pretreatment, the RWB was subjected to dilute alkaline pretreatment using NaOH and KOH (2% w/v). The effect of an oxidizing agent was evaluated by using $H_2O_2$ (0.25 g $g^{-1}$ of biomass). The above mentioned pretreatments were performed in combination with autoclaving at 121°C under 15 psi pressure (i.e.,

physicochemical) with the same incubation time of 30 min. The ratio of the solid phase to liquid phase in each pretreatment was maintained at 1:10. The treated biomass was extensively washed with tap water to remove the impurities. It was then neutralized with distilled water, and dried at 60°C to achieve a constant weight. We investigated the effects of various alkali (NaOH) concentrations (0.5, 1.0, 2.0, or 3.0% w/v), incubation temperatures (30, 100, and 121$^{o}$C), and incubation times (10-60 min) on the hydrolysis of RWB by means of the "one variable at a time" approach while keeping other conditions constant.

**2.3. Acidified sodium chlorite (ASC) and sodium bicarbonate (SB) pretreatment**

The ASC procedure was carried out using 0.4 g sodium chlorite and 0.2 mL acetic acid per gram of dry biomass with intermittent mixing in a fume hood, which was maintained at 80 °C.[20] Fresh acetic acid and sodium chlorite were added each hour until the samples were sufficiently delignified by the persistence of yellowish-green chlorine dioxide gas generated upon mixing of the reagents. The final samples were washed thoroughly with tap water to remove traces of chemicals used during the treatments. As for biomass swelling, the samples were impregnated with sodium bicarbonate 0.5% (w/v) solution for 3 h at room temperature. After autoclaving at 121°C for 15 min, the samples were washed until the solution reached a neutral pH. The NaOH (2%) pre-treated samples were further subjected to delignification treatment using ASC or swelling using SB or the combination of both. Similarly, raw RWB samples were also subjected to individual ASC and SB pretreatment. All samples were dried at 60 °C until a constant weight was achieved and further subjected to enzyme hydrolysis analyses.

**2.4. Enzymatic hydrolysis of pretreated biomass**

RWB pretreated by different methodologies was subjected to enzymatic hydrolysis. Filter paperase activity (FPU) was measured according to IUPAC recommendations using Whatman filter paper as the substrate.[21] One unit of enzyme activity was defined as the

amount of enzyme required to release 2 μmol of reducing sugars per minute. The adsorption capacity of cellulase for cellulose affects the rate of enzymatic hydrolysis of the biomass.[22] Moreover, after hydrolysis of the biomass, the released oligosaccharides and their accumulation cause feedback inhibition of enzymatic activity. To overcome these problems, a two-step enzymatic hydrolysis process was devised that uncouples enzymatic hydrolysis and separation.[12] Two-step enzymatic hydrolysis of untreated and pretreated RWB was performed at the level of 2.0% (w/v) in 20 mL of 50 mM citrate buffer (pH 5.0) containing 0.005% (w/v) sodium azide. An enzyme solution equivalent to a FPU activity of 20 IU $g^{-1}$ of untreated and pretreated substrate was added to Erlenmeyer flasks and the procedure was performed as previously reported.[12] We assessed the effects of increasing alkaline pretreated RWB concentration (5 to 30 g $L^{-1}$) while keeping the enzyme concentration constant (20 FPU $g^{-1}$ of RWB), as well as the effect of increasing enzyme concentrations (5 to 25 FPU $g^{-1}$ of RWB) by keeping the substrate concentration (10 g $L^{-1}$) constant. For evaluating multiple effects including NaOH + $H_2O_2$, NaOH + ASC, NAOH + SB, and NaOH + ASC + SB pretreated biomass hydrolysis, an enzyme dose of 10 IU $g^{-1}$ of substrate was used. The reducing sugars that were released during the two steps of enzymatic hydrolysis were combined to calculate the overall hydrolysis yield.[22]

**2.5. Fermentation medium and conditions for ethanol production**

*Saccharomyces cerevisiae* SR8 was cultured in medium containing glucose (40 g), yeast extract (5.0 g), and peptone (5.0 g) per liter of distilled water.[17] The enzymatic hydrolysates of RWB were concentrated to a reducing sugar concentration of 5% by evaporation in a rotatory evaporator (at constant temperature 80 °C). Filtration-sterilized different pretreated enzymatic RWB hydrolysates were added to minimal medium [(g $L^{-1}$): yeast extract, 5.0; $(NH_4)_2SO_4$, 10.0; $KH_2PO_4$, 4.5; and $MgSO_4.7H_2O$, 1.0] in Erlenmeyer flasks to achieve a final sugar concentration of (20 g $L^{-1}$). These flasks were inoculated with 1%

of 18 h old seed culture (cell density of 0.3 g $L^{-1}$) of *S. cerevisiae* SR8 (washed with 0.1% peptone twice), and were incubated for 48 h at 30°C under shaking conditions (100 rpm) to maintain an oxygen-limited condition. Samples (1 mL) were withdrawn at specific time intervals and centrifuged (7500 *g*, 15 min) to measure the optical density at 600 nm, pH, ethanol concentration, and residual sugar content. The effect of increasing concentrations of NaOH + ASC + SB enzymatic hydrolysates (20, 40 and 50 g $L^{-1}$) on growth and ethanol production was evaluated. The ethanol yield to consumed sugar (g $g^{-1}$) was calculated as the ratio of ethanol to consumed sugar amount ($S_o$–$S_f$, $S_o$: initial sugar concentration and $S_f$: final sugar concentration). The ethanol volumetric productivity (g $L^{-1}$ $h^{-1}$) was calculated as the ratio of ethanol concentration (g $L^{-1}$) at the end of the run to the fermentation time (t, hours). The efficiency of sugar conversion to ethanol (%) has been estimated by the ratio of ethanol yield to the theoretical value of ethanol yield (0.51 g $g^{-1}$ of sugar).

**2.6. Analytical methods**

The chemical composition (cellulose, hemicellulose, and lignin) of raw and pretreated RWB was estimated using the protocol described by Goering and van Soest, (1970).[23] The reducing-sugar content of the enzymatic hydrolysates was determined using the dinitrosalicylic acid method.[24] The presence of sugars (glucose and xylose) in the filtered (0.2-mm pore size) hydrolysates of untreated and pretreated RWB was detected by high-performance liquid chromatography (HPLC) using an ACME-9000 instrument (Young-Lin Instrument, Seoul, Korea) with a SH1011 column (Shodex, Tokyo, Japan) capable of detecting the refraction index using a previously reported method.[22] Fourier-transform infrared (FTIR) spectroscopy (Agilent, Cary 630; USA) of raw and pretreated RWB was conducted in the mid-infrared region by averaging 32 scans in the range of 400–4000 $cm^{-1}$ at resolution 4 $cm^{-1}$ to detect changes in functional groups. Scanning electron microscopy (SEM) images of the particles coated with platinum were obtained using a JEOL JSM-6360A microscope

(Tokyo, Japan) at an operating voltage of 20 kV as previously described.[16] Samples of the raw and pretreated RWB were analyzed by X-ray diffraction (XRD) using a D2 Phaser tabletop model (Bruker, Germany) operating at 30 kV and 10 mA. The radiation was Cu (1.54 Å) and the grade range was between 10° and 60° with a step size of 0.02°. The crystallinity index of cellulose was calculated according to the peak height method.[25,26]

### 2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-kramer multiple comparisons test. Readings were considered significant when p was $\leq 0.05$.

## 3. Results and discussion

### 3.1. Effect of chemical pretreatment on enzyme digestibility of RWB

Pretreatment is a crucial step for the conversion of lignocellulosic biomass into fermentable sugars. The efficiency of pretreatment was evaluated by measuring total sugars released after enzymatic hydrolysis of the pretreated biomass.

In the present study, we evaluated the suitably of individual chemical pretreatment using alkali, oxidizing agents, ASC, and SB to improve the enzymatic digestibility of RWB. Mechanistically, alkali is believed to be effective in lignin removal and reduction of crystallinity, and is also useful for increasing the internal surface area by swelling of the biomass fibers.[27] In addition, alkaline saponification of acetyl and uronic ester bonds makes the structure more accessible to hydrolytic enzymes.[7] Initially, alkaline pretreatment using NaOH and KOH (2% w/v) were conducted to determine hydrolytic efficiency after the enzymatic reaction. It was observed that alkaline pretreatment with NaOH in combination with autoclaving produced a considerably higher amount of reducing sugars of about 50.5 g per 100 g of biomass with a hydrolysis yield of 69.2% of RWB (Table 1). Alkaline pretreatment with NaOH significantly removed hemicellulose and lignin compared to KOH

pretreatment. Autoclaving with NaOH increases the water holding capacity, and biomass swelling improves enzyme accessibility to the internal structure of RWB, leading to a higher yield of reducing sugar.[28] The main components of the untreated and pretreated biomass with various chemical agents are shown in Table 1. The cellulose content generally increases possibly by higher solubilization of hemicelluloses and structural modification/removal of lignin.[13,29,30] The SEM image of the 2% NaOH pretreated biomass demonstrated an increase in the surface area by rupturing the closed structure of the biomass, which became more rough and porous (Fig. S1 B). Several researchers have recommended mild alkaline pretreatment as an effective method for lignocellulosic biomass including rice straw switchgrass, sorghum straw, corn stover, and wheat straw.[7] Similarly, in our previous study, we also observed that alkaline pretreatment to the rice paddy straw gave better saccharification (703 mg $g^{-1}$ of PS) and hydrolysis yield (84.2%).[12]

We have also investigated the oxidative pretreatment of biomass with the oxidizing agent $H_2O_2$. $H_2O_2$ essentially assists in effective solubilization of lignin and hemicellulose of the biomass.[31] In most cases, the peroxides are transformed *in situ* into hydroxyl radicals ($OH^{\bullet}$), which are far more powerful than the peroxide itself. This action makes the cellulose of the biomass more accessible to enzymatic hydrolysis. $H_2O_2$ pretreatment was not effective for the destruction of RWB. After enzymatic hydrolysis, moderate reducing sugar production of 34.4 g per 100 g of biomass with hydrolysis yield of 53.7% was observed (Table 1). After pretreatment, there were no significant change in the morphological structure of the biomass was observed (Fig. S1 C).

Initially, we evaluated the delignification of raw RWB by acidified sodium chlorite pretreatment. Chlorite is a strong oxidant that acts selectively on lignin.[14] In the delignification process of RWB, lignin was clearly oxidized and removed by chlorite ions. The lignin content of RWB decreased to about 33% (w/w) of the control after ASC

pretreatment and significant changes in the morphological structure of biomass was also observed (Fig. S1 D). After enzymatic hydrolysis, 47.5 g of reducing sugar per 100 g of biomass was produced with 67.8% hydrolysis yield (Table 1). Similarly, studies have demonstrated that ASC treatment effectively removes lignin from the biomass.[20] Treatment with sodium bicarbonate also changes the properties of the cellulose surface.[15] In this case, carboxylation of the cellulose surface supported the disintegration of cellulose structures, and allowed the enzyme to degrade the cellulose micro-fibrils. In our study, SB pretreatment was not effective for the reduction of lignin as well as less effective for saccharification (34.5 g of reducing sugar per 100 g of biomass) and hydrolysis yield (51.5%) (Table 1; Fig S1 E).

**3.2. Optimization of alkaline pretreatment**

Alkaline pretreatment (NaOH) was more effective for the disruption of RWBand better saccharification compared to other individual chemical pretreatments (Table 1). Based on this result, NaOH alkaline pretreatment was further optimized for better saccharification yield. We studied the effects of various alkali (NaOH) concentrations, incubation temperatures, and incubation times on the hydrolysis of RWB. We also assessed the effect of increasing RWB concentration, as well as the effect of increasing enzyme concentration on the hydrolysis of RWB. The optimum pretreatment condition was observed at 2% NaOH by autoclaving at 121$^{o}$C for 30 min (Fig. S2 A, B and C). The maximum hydrolysis yield with sugar production from RWB was obtained by using 10 g $L^{-1}$ biomass with 20 FPU $g^{-1}$ of RWB enzyme dosage (Fig. S3 A and B). These optimal conditions were used in further experiments.

**3.3. Effects of multiple pretreatment on RWB hydrolysis**

Biomass lignin is arranged in multiple lamellar sheets of lignocellulose matrices, thus a single batch reaction of chemical pretreatment is not sufficient to achieve complete biomass delignification.[13,14] To achieve maximum delignification we have used NaOH alkaline

pretreated biomass for sequential pretreatments with $H_2O_2$, ASC, SB, or ASC + SB to enhance the saccharification of RWB using a low enzyme dose.

### *3.3.1. Effects of NaOH + $H_2O_2$ pretreatment on RWB hydrolysis*

$H_2O_2$ had significant effect on biomass pretreatment in an alkaline environment (pH > 10).[5,31] Following enzymatic hydrolysis of NaOH + $H_2O_2$, pretreated biomass showed significantly higher RS yield (55.5 g per 100 g of biomass) with better hydrolysis yield 73.0% than alkaline pretreatment alone (Table 2). These results are clear evidence of the beneficial action of $H_2O_2$ during pretreatment, as previously mentioned.[31] The effect of $H_2O_2$ pretreatment on chemical composition and structure of biomass are evidence of the significant removal of lignin (Fig. S1 F). In our case, a lower concentration of $H_2O_2$ was effective for lignin removal as well as for better hydrolysis yield from RWB. Similar observations during the pretreatment of various lignocellulosic biomass using oxidizing agents have also been reported.[31,32]

### *3.3.2. Effect of* NaOH *pretreatment combined with ASC and SB on RWB hydrolysis*

Pre-treatment using acidified sodium chlorite successfully improved the efficiency of saccharification of RWB by effectively removing lignin, compared to the control (Table 1). It was reported that ASC pretreatment was effective against amorphous cellulose but had no detectable effect on digestibility or accessibility of crystalline cellulose.[33] In NaOH + ASC pretreated biomass, a significant reduction in lignin and hemicellulose, and a sharp increase in cellulose content were observed (Table 2). After enzymatic hydrolysis, reduced sugar production (66.8 g per 100 g of biomass) and increased hydrolysis yield (85.6%) were observed, which was significantly higher than individual NaOH or ASC pretreated RWB (Tables 1 and 2). Interestingly, much higher rates of saccharification (90.6%) were achieved when SB treatment was additionally applied after chlorite treatment (Table 2). Therefore, the swelling seems to play an important role, not only in the removal of surface lignin, but also in

the removal of integrated lignin. In contrast, sequential NaOH + SB pretreatment was not effective, considering that the hydrolysis yield (78.8%) and sugar production (57.5 g per 100 g of biomass) were lower than NaOH + ASC (85.6% and 66.8 g per 100 g of biomass) and NaOH + ASC + SB (90.6% and 72.5 g per 100 g of biomass) pretreatment (Table 2). It is noteworthy that this improved saccharification could be achieved using half the enzyme dose compared to individual pretreatment. In addition, it also increased the potential application of multiple pretreatments. Therefore, these results indicate that the sequential multiple pretreatment processes were very effective for increasing the accessibility of the surface of RWB to enzymes, thereby raising the rate of enzymatic hydrolysis. The SEM image clearly showed that with NaOH + ASC, NaOH + SB, and NaOH + ASC + SB pretreatment, the integrated lignin was almost completely removed from cellulose structures by the swelling treatment, such that cellulose fibrils appear to be separated and accessible to the enzymes (Fig S1 G, H, and I).

Even though these sequential pretreatment methods showed significant delignification, no complete removal of sugars from NaOH + ASC + SB pretreated RWB was observed. One possible explanation for this phenomenon is that adjacent hemicellulose-free cellulose micro-fibrils are aggregated upon elimination of the lignin spacer.[34,35] Moreover NaOH + ASC + SB pretreatment resulted in reduced sugar production of 725 mg $g^{-1}$ of RWB with 90.6% hydrolysis yield, which was better than or comparable to most of the reported results from relevant studies. These results emphasize the potential of using NaOH + ASC + SB pretreatment of RWB as effective feedstock for biofuel synthesis.

In this study we have utilized whole rice plant body except root for the preparation of biomass feedstock. Complex multicellular structures of plant tissues including leaves, stems, and cobs respond differently to pretreatment and saccharification. For example, the stem part (ground tissues) is more resistant for hydrolysis than upper portion (vascular tissues) due to

its rigid architecture.[36] For better delignification and enzymatic digestibility of whole RWB, a single batch of alkaline pretreatment was not effective enough. The sequential pretreatment improved the delignification and saccharification yield compared to individual alkaline pretreatment from 33.3% and 69.2% to 76.7% and 90.6%, respectively. Especially, the foregoing results suggest that the sequential pretreatment was effective in total reducing sugar yield from whole RWB with very low enzyme dosage (10 FPU) compared to other studies (Table S1). However further research is needed on the recovery and reusability of chemicals to reduce the cost of pretreatment process.

**3.4. FTIR analysis of pretreated RWB**

FTIR has been used to investigate the changes in functional groups observed in raw RWB during various chemical treatments. Figure 1A shows the FTIR spectra of individual chemical pretreatments and Figure 1B shows the spectrum of sequential multiple pretreated RWB. The effect of different chemical treatment processes on the composition of RWB was evaluated in terms of relative changes in absorbance at specific band positions. The relative intensity changes of various bands after the pretreatment processes are summarized in Table 3. Relative percentage changes with positive values indicate the reduction of a component relative to the control band, suggesting that the majority of absorption peaks were reduced by all types of chemical treatment (Table 3). Especially, significant reductions of a few peaks were observed for the sequential multiple pretreatments, such as in lignin peaks of 1457 $cm^{-1}$ related to aromatic ring vibration and 1743 $cm^{-1}$ related to lignin side chains and linkages between carbohydrate and lignin.[5,29] The reductions of those peaks with NaOH + ACS were more significant than those with NAOH, indicating better delignification of RWB by sequential pretreatment (Table 3). Moreover significant reductions in peaks of 1230 $cm^{-1}$ and 1376 $cm^{-1}$ representing hemicellulose-lignin linkages and C=O adsorption of acetyl group suggest the cleavage of hemicellulose from lignin during NaOH + ASC. [5,29] It was supposed

that during ASC pretreatment the chlorine species, such as chlorite ($ClO_2^-$) ion, were effectively oxidized and removed lignin by breaking linkages between hemicellulose and lignin,[14] which was supported by FTIR data. Sequential NaOH + ASC + SB pretreatment showed further reductions in lignin peaks of 1457 $cm^{-1}$ and 1743 $cm^{-1}$ as well as in hemicellulose peaks of 1230 $cm^{-1}$ and 1376 $cm^{-1}$ compared to NaOH + ASC (Table 3), which indicates more lignin and hemicellulose were removed.

The peak at 2,900 $cm^{-1}$ is due to asymmetrical stretching of $CH_2$ and CH denotes the characteristics of cellulose (Fig. 1 A-B). The region between 3,800 and 3,000 $cm^{-1}$ covers the related crystalline structure of cellulose (Fig. 1 A-B). Bands in this region do not seem to change much during individual and multiple pretreatments. Interestingly, broadening of peaks in cellulose related region between 3,800 and 3,000 $cm^{-1}$ was noticeable after NaOH + ASC + SB compared to NaOH + ACS, indicating swelling of delignified biomass. It has been suggested that swelling of biomass increases the enzyme accessibility towards cellulose micro-fibrils.[14] Based on FTIR spectra analysis, sequential multiple pretreatment showed very effective degradation of lignin and hemicelluloses as well as swelling of cellulose structure, which led to higher saccharification using a low enzyme dose.

### 3.5. X-ray diffraction studies of pretreated RWB

XRD analysis is a useful technique to determine the crystallinity index (CrI) of the biomass. During pretreatment, non-crystalline or amorphous components of biomass such as lignin and hemicellulose are removed.[25] Increase in the CrI is essentially an indication of higher cellulose content of biomass. Among several factors, it was observed that crystallinity significantly affects enzymatic saccharification of glucan.[27] XRD diffractograms of RWB treated with individual chemicals showed an increase in the crystallinity index (CrI) in NaOH (57%), ASC (58%), and $H_2O_2$ (56%) than that of the untreated RWB (43%). In SB individual

pretreatment an increase in the CrI was also observed, but to a lesser extent (Table 1). However, a significant increase in CrI was observed with multiple pretreated RWB. Particularly in the cases of NaOH + ASC and NaOH + ASC + SB, major increase in CrI, which was about 63% and 66%, respectively was observed. However, in the cases of NaOH + $H_2O_2$ and NaOH + SB, a moderate increase in CrI was observed (Table 2). The results suggest that after multiple pretreatments, significant removal of hemicellulose and lignin occurs and exposes the cellulosic fraction of biomass with high crystalline character. This cellulose component becomes more accessible to the hydrolysis reaction leading to a better saccharification yield. The actual diffractograms after different treatments are shown in Figure 2A-B. Similar observations have previously been reported.[26,28,37] Moreover, this increase in CrI suggests that pretreatment significantly affects the amorphous portion relative to the crystalline portion of the biomass.[5]

**3.6. Ethanol production using RWB enzymatic hydrolysates**

The potential of RWB enzymatic hydrolysates from different pretreatments has been assessed for fermentative ethanol production by using the developed *Saccharomyces cerevisiae* SR8 strain. *Saccharomyces cerevisiae* SR8 showed significant consumption of sugars from pretreated NaOH, NaOH + ASC, and NaOH + ASC + SB from RWB enzymatic hydrolysates (each 20 g $L^{-1}$ sugar). In each treated biomass hydrolysate without detoxification, sugar consumption of greater than 90% with higher ethanol yield was reported (Table 4). Importantly, the sugar consumption and ethanol yield was similar to that of the mixture of synthetic sugars (data not shown). The maximum sugar consumption was approximately 95%, the ethanol yield was approximately 46.5 g per 100 $g^{-1}$ of sugar, and the sugar conversion to ethanol was approximately 91.33% when we utilized NaOH + ASC + SB pretreated RWB enzymatic hydrolysates. In pretreated biomass, the observed low sugar consumption may be due to the presence of small amounts of arabinose and oligosaccharides. The fermentation of

RWB hydrolysates with increasing concentrations led to a continuous increase in biomass, sugar consumption, and ethanol production (Fig. 3). However at a higher concentration (50 g $L^{-1}$), a small reduction in ethanol production was observed, which needs further investigation. According to these results, we conclude that NaOH + ASC + SB is effective in enhancing the enzymatic saccharification of RWB and ethanol production. This process could reduce ethanol production costs and be used for the bioconversion of all carbohydrates present in various lignocellulosic biomasses. Successful conversion of such waste biomasses to ethanol would not only help to solve pollution problems but also create new revenue sources. However further research is needed for simultaneous saccharification and fermentation approach by developing microbial strain having ability to produce cellulase and ethanol, which could enhance the commercial applicability of the process.

## 4. Conclusion

In this study, we demonstrate the potential of RWB feedstock for biofuel production. We evaluated different pretreatment methods and investigated structure of biomass after pretreatment and its relation to hydrolysis. Among all the pretreatment techniques, NaOH + ASC + SB pretreated RWB produced a maximum reducing sugar of 72.5 g per 100 g of biomass with 90.65% hydrolysis yield using a low enzyme dose (10 FPU $g^{-1}$ of biomass). The resulting biomass hydrolysates produced better ethanol yield, implying the applicability for commercial ethanol production. The developed sequential multiple pretreatment can be an attractive pretreatment method for other lignocellulosic biomass.

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**Figure legends:**

**Fig. 1.** Chemical changes in rice waste biomass (RWB), as determined by FTIR. (A) after individual chemical pretreatment and (B) after sequential multiple chemical pretreatments.

**Fig. 2.** X-ray diffraction pattern of rice waste biomass (RWB). (A) after individual chemical pretreatment and (B) after sequential multiple chemical pretreatment.

**Fig. 3.** Effect of increasing NaOH + ASC + SB enzymatic hydrolysate concentration (20-50 g $L^{-1}$) on (A) growth and (B) fermentation parameters using the *Saccharomyces cerevisiae* SR8 strain.

**Table 1** Effects of different individual pretreatments on the chemical composition and hydrolysis of whole rice waste biomass.

| Pretreatment methods | Pretreatment conditions | Biochemical composition (%) | | | TRS after enzymatic hydrolysis (g/100 g of biomass) | Hydrolysis yield (%) | CrI (%) |
|---|---|---|---|---|---|---|---|
| | | Cellulose | Hemicellulose | Lignin | | | |
| Control | No pretreatment | 35.1 ± 0.85 | 30.2 ± 0.45 | 18.0 ± 0.35 | 8.50 ± 0.70 | 13.1 ± 0.84 | 43 |
| Chemical pretreatment | | | | | | | |
| Alkali | 2% (w/v) NaOH, 121°C, 30 min | 55.0 ± 0.88 | 18.2 ± 0.48 | 12.0 ± 0.32 | 50.5 ± 3.15 | 69.2 ± 0.84 | 57 |
| | 2% (w/v) KOH, 121°C, 30 min | 48.2 ± 0.71 | 19.1 ± 0.55 | 14.1 ± 0.34 | 40.5 ± 3.65 | 60.4 ± 0.88 | ND |
| $H_2O_2$ | $H_2O_2$ (0.25 g $g^{-1}$ of biomass), 121 °C, 30 min | 46.1 ± 0.84 | 18.1 ± 0.38 | 14.1 ± 0.62 | 34.4 ± 2.15 | 53.7 ± 0.77 | 56 |
| ASC | 0.4 g sodium chlorite and 0.2 mL acetic acid $g^{-1}$ dry biomass at 80°C | 52.1 ± 0.88 | 18.2 ± 0.48 | 12.0 ± 0.44 | 47.5 ± 3.20 | 67.8 ± 0.78 | 58 |
| SB | 0.5% (w/v), 121°C, 15 min | 45.0 ± 0.76 | 22.1 ± 0.55 | 13.2 ± 0.53 | 34.5 ± 2.20 | 51.5 ± 0.88 | 50 |

TRS: total reducing sugar; CrI: crystalline index; ND: not determined;
Values are the mean of three experiments ± SEM. Statistics were determined by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test.

**Table 2** Synergistic effect of multiple pretreatments on the chemical composition and hydrolysis of whole rice waste biomass.

| Pretreatment method | Pretreatment conditions | Biochemical composition (%) | | | TRS after enzymatic hydrolysis (g/100 g of biomass) | Hydrolysis yield (%) | CrI (%) |
|---|---|---|---|---|---|---|---|
| Multiple pretreatment | | Cellulose | Hemicellulose | Lignin | | | |
| Control | No pretreatment | 35.1 ± 0.85 | 30.2 ± 0.45 | 18.0 ± 0.35 | 8.50 ± 0.70 | 13.1 ± 0.84 | 43 |
| NaOH | 2% (w/v) NaOH, 121°C, 30 min | 55.0 ± 0.88 | 18.2 ± 0.48 | 12.0 ± 0.32 | 50.5 ± 3.15 | 69.2 ± 0.88 | 57 |
| NaOH + $H_2O_2$ | 2% (w/v) NaOH + $H_2O_2$ (0.25 g/g of biomass) 121°C, 30 min | 62.1 ± 0.76 | 14.2 ± 0.35 | 8.10 ± 0.32 | 55.5 ± 3.50 | 73.0 ± 1.12 | 58 |
| NaOH + ASC | NaOH pretreated biomass + 0.4 g sodium chlorite and 0.2 mL acetic acid $g^{-1}$ dry biomass at 80°C | 72.2 ± 0.78 | 6.15 ± 0.32 | 5.15 ± 0.32 | 66.8 ± 4.80 | 85.6 ± 1.24 | 63 |
| NaOH + SB | NaOH pretreated biomass + $NaHCO_3$ 0.5% (w/v), 121°C, 15 min | 62.1 ± 0.72 | 11.4 ± 0.51 | 9.10 ± 0.42 | 57.5 ± 3.60 | 78.8 ± 1.45 | 60 |
| NaOH + ASC + SB | NaOH pretreated biomass + 0.4 g sodium chlorite and 0.2 mL acetic acid $g^{-1}$ dry biomass at 80°C + $NaHCO_3$ 0.5% (w/v), 121°C, 15 min | 75.1 ± 1.15 | 5.10 ± 0.31 | 4.20 ± 0.25 | 72.5 ± 4.65 | 90.6 ± 2.22 | 66 |

TRS: total reducing sugar; CrI: crystalline index
Values are the mean of three experiments ± SEM. Statistics determined by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test.

**Table 3** Characterization of pretreated biomass in terms of relative change in intensity (%)[a] by FTIR spectroscopy

| Band position ($cm^{-1}$) | Assignment[b] | $H_2O_2$ | SB | ASC | NaOH | NaOH +$H_2O_2$ | NaOH +SB | NaOH +ASC | NaOH +ASC +SB |
|---|---|---|---|---|---|---|---|---|---|
| 1057 | C=O stretching due to carbohydrate–lignin linkage | -6.70 | 4.90 | -4.80 | -6.70 | 7.31 | -4.87 | 3.35 | 9.75 |
| 1230 | Hemicellulose–lignin linkage | 19.8 | 9.45 | 19.6 | 17.6 | 21.9 | 20.5 | 24.6 | 32.9 |
| 1376 | Band of hemicellulose | 23.8 | 7.30 | 24.6 | 23.2 | 19.1 | 24.6 | 26.0 | 31.5 |
| 1457 | Aromatic ring vibration (related to lignin removal) | 6.20 | 3.95 | 6.75 | 6.80 | 5.60 | 6.74 | 7.86 | 8.42 |
| 1743 | Carbonyl bonds (related to lignin side chain removal); C=O stretching due to carbohydrate linked with lignin | 65.3 | 22.9 | 67.3 | 66.7 | 55.8 | 67.3 | 78.1 | 86.5 |
| 2934 | C-H stretching (related to rupture of methyl/methylene group of cellulose) | 4.70 | 2.35 | 8.20 | 5.30 | 8.23 | 8.23 | 8.00 | 12.9 |
| 3026 | Vibration of valence bands of -H- bands of the OH group and the bands of intra-molecular and intermolecular –H-bands | 9.00 | 3.60 | 9.60 | 8.50 | 9.60 | 10.2 | 10.0 | 15.7 |
| 3339 | O-H stretching (related to rupture of cellulose hydrogen bonds) | -2.90 | 5.75 | 1.45 | -2.30 | -2.30 | 1.45 | -8.00 | 4.00 |

[a] % Relative change = 100 × (intensity of untreated solid − intensity of pretreated solid) ⁄ intensity of untreated solid, where a positive number indicates reduction

[b]Adopted from (Singh et al., 2014; Saratale et al., 2014; Adapaa et al., 2009; Kumar et al., 2009).

**Table 4** Fermentation parameters of enzymatic hydrolysates of different pretreatment of rice waste biomass by the *S. cerevisiae* SR8 strain

| RWB hydrolysates | Initial sugar concentration ($g\ L^{-1}$) | Sugar consumption (%) | Ethanol ($g\ L^{-1}$) | Volumetric ethanol production ($g\ L^{-1}\ h^{-1}$) | Ethanol yield ($g\ g^{-1}$) |
|---|---|---|---|---|---|
| NaOH | 20 | 90 ± 0.85 | 8.35 ± 0.55 | 0.23 ± 0.01 | 0.463 ± 0.01 |
| NaOH+$H_2O_2$ | 20 | 94 ± 0.92 | 8.65 ± 0.54 | 0.24 ± 0.02 | 0.460 ± 0.02 |
| NaOH+ASC | 20 | 95 ± 0.88 | 8.82 ± 0.61 | 0.25 ± 0.01 | 0.464 ± 0.02 |
| NaOH+SB | 20 | 94 ± 0.92 | 8.75 ± 0.64 | 0.24 ± 0.01 | 0.465 ± 0.02 |
| NaOH+ASC+SB | 20 | 95 ± 0.85 | 8.85 ± 0.63 | 0.25 ± 0.02 | 0.465 ± 0.03 |
| Mixture of sugars | 20 | 94 ± 0.85 | 8.84 ± 0.55 | 0.24 ± 0.02 | 0.470 ± 0.03 |

Values are the mean of three experiments ± SEM. Statistics were determined by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test

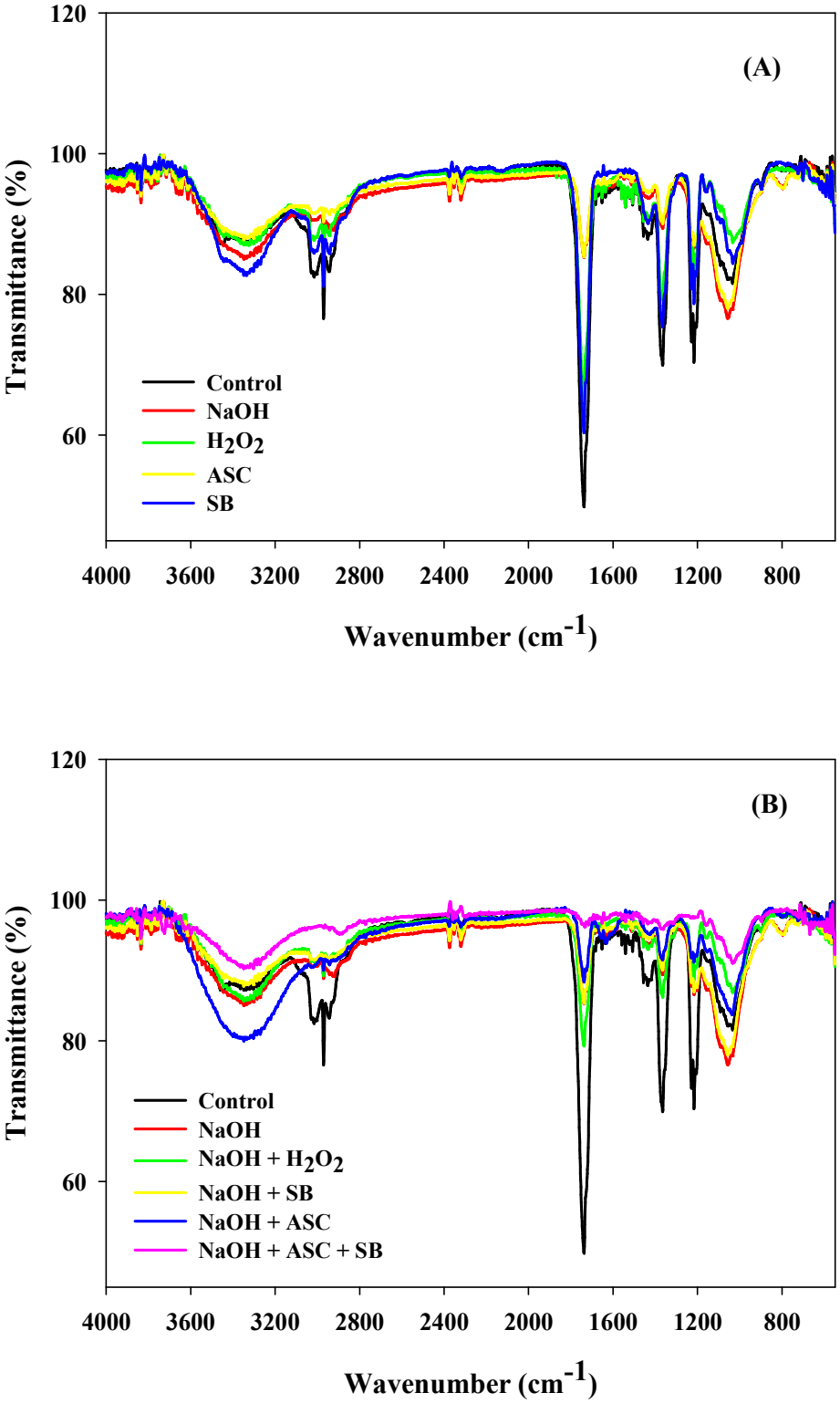


**Fig. 1**

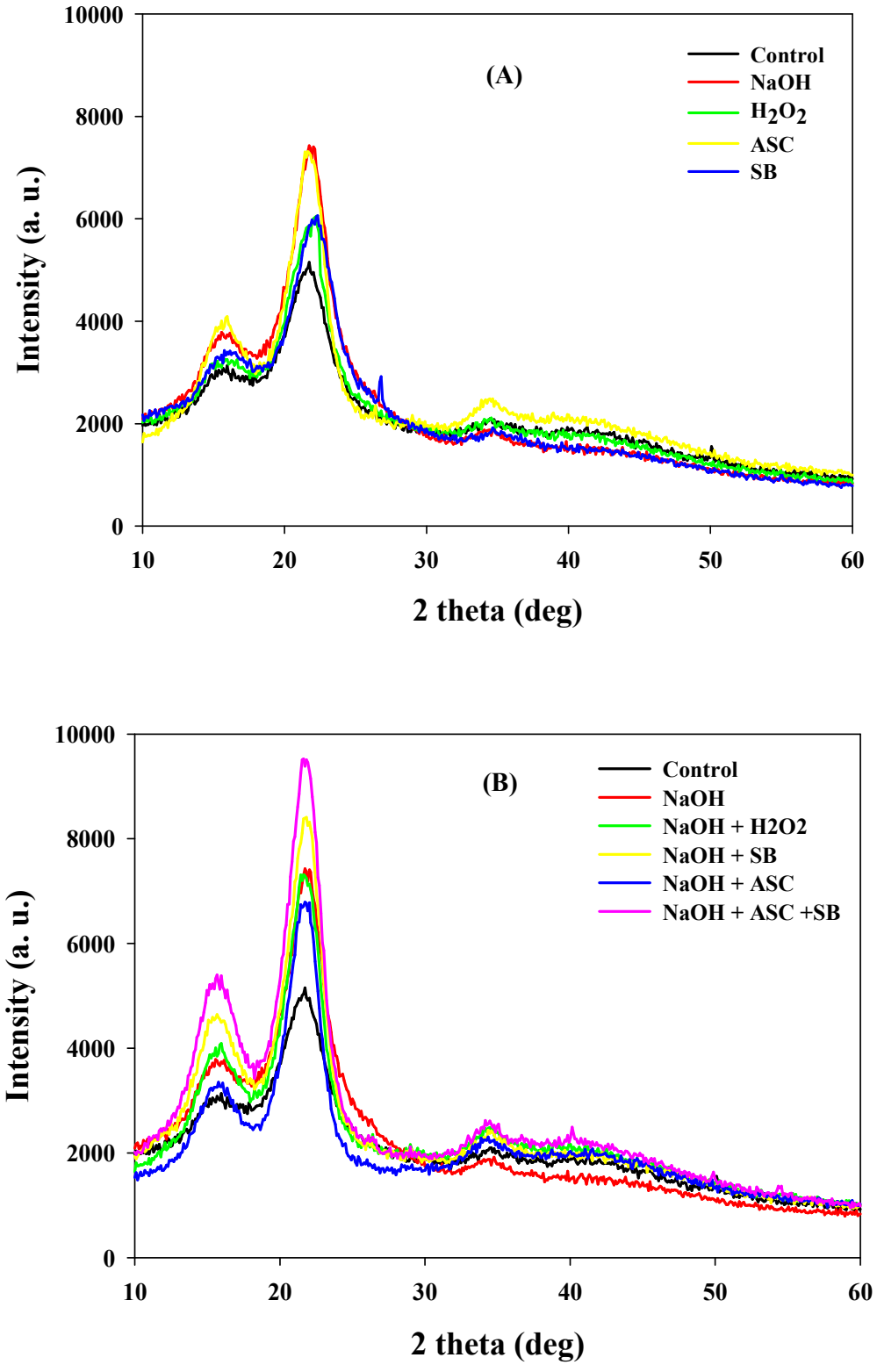


Fig. 2

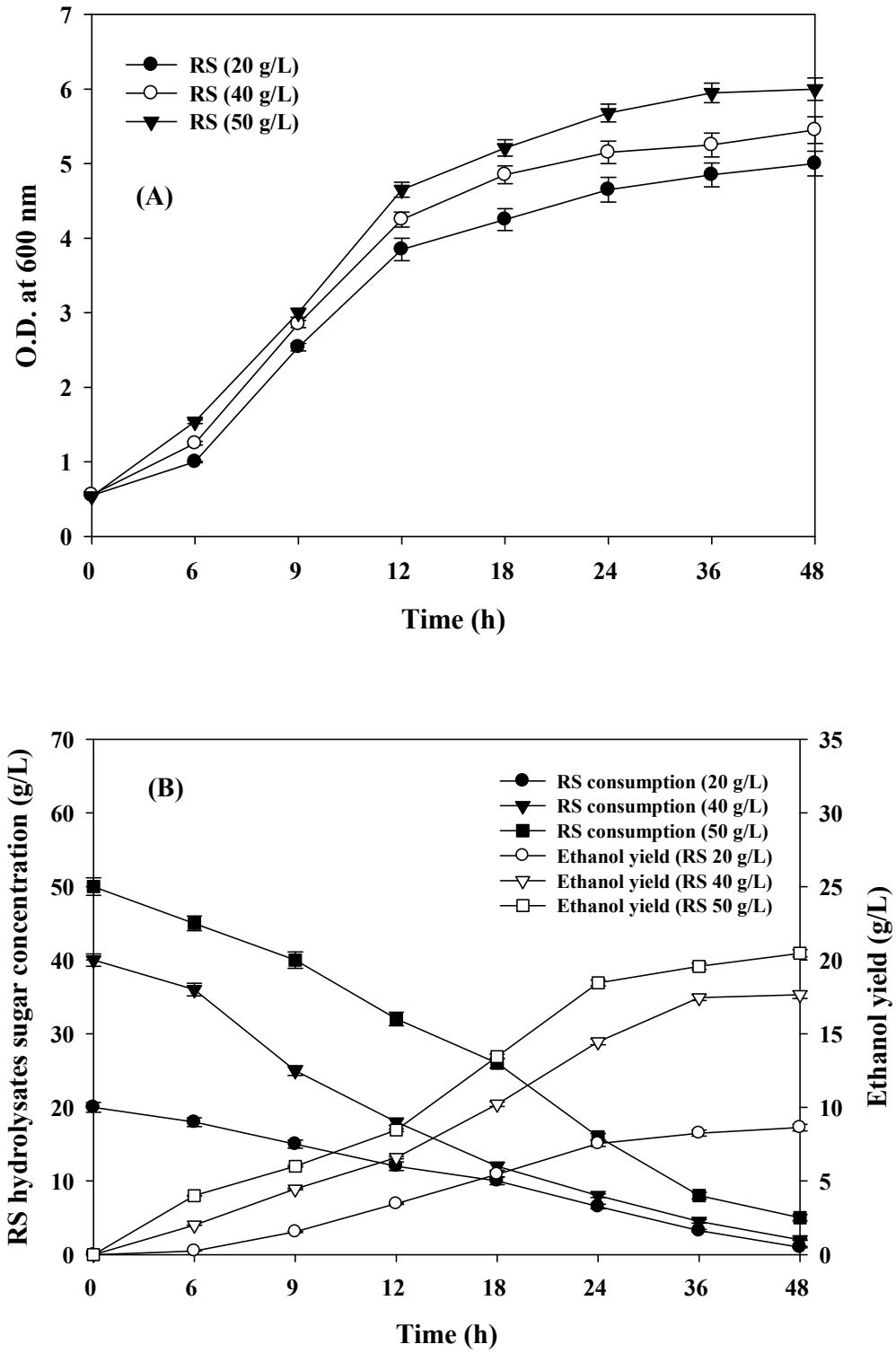


**Fig. 3**