

Recovery of carbon from rice straw for simultaneous production of protein, lipid and carbohydrate by *Scenedesmus* sp. via mixotrophic cultivation

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Abstract. Rice straw is abundantly generated as a by-product of agriculture in Viet Nam. However, the material mainly contains hemicellulose and cellulose, which can be hydrolyzed to reducing sugars as a carbon source for the mixotrophic production of protein-rich microalgae biomass. In this study, rice straw was obtained from local farmers and transformed to hydrolysate via separated alkaline or acid and sequential alkaline-acid treatments to evaluate sugar conversion efficiency. The hydrolysate then was used as a carbon source for the cultivation of *Scenedesmus* sp. via mixotrophic mode. Data revealed that pretreatment with H₂SO₄, NaOH, and combined NaOH + H₂SO₄ yielded sugar conversion of 12 – 13 %, 11 – 12 % and 22 %, respectively. *Scenedesmus* sp. displayed a good growth performance in both rice straw hydrolysates with and without supplement of nitrogen and phosphorous, reaching the maximal optical density of 1.5 Abs in the culture medium of BG-11 with 10 – 50 % v/v hydrolysate. The sugar utilization efficiency by *Scenedesmus* sp. was determined as 70 – 94 %. The *Scenedesmus* sp. was assayed to be rich in protein with its content of up to 45 % based on a dry basis. The *Scenedesmus* sp. biomass is a potential protein source for animal and aquafeed formulation. Our preliminary results demonstrated that recovery of carbon from such agricultural by-products for protein-rich material for novel food development in animal food and aquafeed industries is promising.

Keywords: Biomass production, Carbon, protein, rice straw hydrolysate, *Scenedesmus* sp.

Classification numbers: 1.4.4, 1.4.3, 3.1.1

1. INTRODUCTION

Rice straw is greatly generated as a popular agricultural byproduct in Viet Nam. According to a recent study published in 2017, Viet Nam produced approximately 70.7 million dry tons of rice straw [1]. Rice straw in Viet Nam is a huge renewable resource, which is promising for application in many fields. In the past, rice straw was mainly used for burning, roofing, and

feeding livestock (e.g., cow, horse, and buffalo). According to the International Rice Research Institute (IRRI) there were about 20 million tons of rice straw burned in Viet Nam per year accounting for 60 % of the total volume which not only wasted natural resources, but also caused environmental pollution due to the emission of greenhouse gases (e.g., CO₂, CO, CH₄, SO₂, NO₂) [2]. Moreover, rice smoke is spicy, causing tears and stimulating throats. People who breathe rice straw burning smoke are easily cough, have sneeze, nausea, and suffocate, which affects health and pollutes the air environment [2].

Viet Nam was committed with COP26 to reduce CO₂ emission and reach net-zero emission for the entire national economy by 2050. Therefore, the research trend is strongly encouraged toward reducing carbon emissions and achieving a circular economy by various research directions including the utilization of biomass and transforming biomass/agricultural wastes in sustainable pathways.

Rice straw mainly contains cellulose, hemicellulose and lignin which are generally accounted for 32 - 47, 19 - 27, and 5 - 24 %, respectively [3]. Among these components, cellulose and hemicellulose can be used as carbon sources for chemical production via biological processes [4]. However, to be utilized as a carbon source, cellulose- and hemicellulose-based rice straws need to be pretreated by chemical and/or biological methods to generate reducing sugars. Recently, rice straw have received considerable attention from numerous domestic scientists to study and explore their potential for application in many areas such as materials (e.g., silica and lignin, etc.) and biofuel production (e.g., ethanol, etc.) [5]. A new research direction is to use microalgae for the production of green biomass under mixotrophic/heterotrophic mode [6, 7]. The green algal biomass can be refined to produce various chemicals (biodiesel, ethanol), materials (bioplastic), bioactive compounds (lutein, chlorophylls) and feed and food [6, 8].

Scenedesmus is a green unicellular alga, widely distributed in freshwater and soil, high growth rate and is capable of heterotrophic/mixotrophic growth in organic carbon-containing media, thus *Scenedesmus* microalgae biomass is suitable for mass production [9]. Moreover, lignocellulosic hydrolysate from rice straw will be a potential carbon source for algal biomass production. Recently, *Scenedesmus* has been reported as a microalgal accumulating high protein and lipid content, which is a promising source for animal and aquafeed production [10]. More importantly, *Scenedesmus* has recently been reported to display a capability in the utilization of xylose at a rate similar to that of glucose utilization [11], demonstrating the potential strain for simultaneous conversion of five-carbon and six-carbon monosugars to high-value biochemical products.

Therefore, the objectives of this study are (i) to investigate chemicals-assisted treatment method for rice straw to produce reducing sugars, (ii) to evaluate the growth of *Scenedesmus* sp. in the hydrolysates, and (iii) to assess the protein and other biochemical contents of *Scenedesmus* sp. for potential application in animal and aquafeed production.

2. MATERIALS AND METHODS

2.1. Materials

Rice straw was purchased from the local community at Tay Tuu commune, Bac Tu Liem district, Hanoi City, Viet Nam. It was cut into 2 - 3 cm long-size and dried in an oven at 100 °C until constant weight. The dried rice straw was ground into microsize powder using a mini-mill (800A, LaLiFa Co., Ltd, Viet Nam) for hydrolysis study. The chemical composition of rice

straw including cellulose, hemicellulose, lignin, protein and ash were determined as 43, 25, 12, 3 - 4 and 16 – 17 %, respectively (see Section 2.3.2).

Microalgae strain *Scenedesmus* sp. was obtained from Algae Collection at Department of Green Chemistry, Institute of Chemistry, Vietnam Academy of Science and Technology and maintained on the solid agar BG-11 medium. The BG-11 medium was consisted of (g/L) NaNO₃, 1.5; K₂HPO₄, 0.04; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.036; Citric acid, 0.006; Ferric ammonium citrate, 0.006; EDTA (Ethylenediaminetetraacetic acid), 0.001; Na₂CO₃, 0.02; mix A5 solution, 1 mL/L; agar, 10. Mix A5 consists of H₃BO₃, 2.86 g/L; MnCl₂·4H₂O, 1.81 g/L; ZnSO₄·7H₂O, 0.222 g/L; Na₂MoO₄·2H₂O, 0.39 g/L; CuSO₄·5H₂O, 0.079 g/L; Co(NO₃)₂·6H₂O, 0.0494 g/L. The algal culture was routinely transferred from the solid agar to 250 mL flask containing 150 mL liquid BG-11 medium and controlled growth under the conditions of shaking at 50 rpm on a shaker (No. 3006, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany), continuous illumination at 6600 lux and temperature of 25 ± 0.5 °C for 7 days to achieve a seed culture with optical density (740 nm) of 1.0 Abs. The seed culture was used for growing *Scenedesmus* sp. in rice straw hydrolysate.

2.2. Methods

2.2.1. Pretreatment of rice straw

Rice straw powder was pretreated by chemical-assisted hydrolysis using NaOH and H₂SO₄. For H₂SO₄ treatment, 10 g rice straw powder was mixed with 50 ml H₂SO₄ 10 % and 350 ml deionized water (solid loading of 2.5 %) in a glass bottle followed by heat treatment at 121°C for 20 min in an autoclave (LS-B75L, Jibimed, Jiangsu, China). After the heat treatment was completed, the glass bottle was cooled down to room temperature and the rice straw mixture was filtered via a membrane to separate the treated rice straw and filtrate. The treated rice straw was washed with deionized water until neutral pH was reached, followed by drying at 105 °C for 48 h to obtain a dryness. The filtrate was collected, and neutralized with CaO for reducing sugar determination.

For NaOH treatment, 10 g rice straw powder was mixed with 50 ml NaOH 10N and 350 ml deionized water in a glass bottle followed by heat treatment at 100 °C for 2 h in an oil bath. After the heat treatment was completed, the glass bottle was cooled down to room temperature, and the rice straw mixture was filtered via a membrane to separate the treated rice straw and filtrate. The treated rice straw was washed with deionized water until neutral pH was reached followed by drying at 105 °C for 48 h to obtain dryness. The filtrate was collected, treated with H₂SO₄ 10 % and reducing sugar determination.

For NaOH and H₂SO₄ combined treatment, the rice straw powder was firstly treated with NaOH 10 N to obtain the dryness, which was followed by H₂SO₄ 10 % treatment as described above. The filtrates obtained from NaOH and H₂SO₄ treatments were neutralized by H₂SO₄ and CaO, respectively, for determination of total reducing sugars. The treated rice straw was washed with deionized water to neutral pH and dried according to the conditions mentioned above to obtain dryness.

2.2.2. Cultivation and harvesting of Scenedesmus sp. in rice straw hydrolysate

The rice straw hydrolysate after neutralization to pH = 7 was diluted with water at the ratio of 10 %, 25 %, and 50 %. The seed culture of *Scenedesmus* sp. (10 % v/v) was inoculated into a

500 ml flask containing 200 ml sterilized hydrolysate. The microalgal culture was constantly shaken at 150 rpm on a shaker under continuous light illumination (6600 lux), a temperature of 25 °C for ten days. After stopping the algal culture, the algal culture was filtered to obtain the filtrate for determination of residual reducing sugar content. The algal biomass was harvested by centrifugation at 4000 rpm for 10 min using a centrifuge (Z326K, HERMLE Labortechnik GmbH, Wehingen, Germany) and washed with deionized water for several times to remove nutrient impurities. The wet biomass was lyophilized for 48 h to obtain solid form which then was manually ground to microsize powder. The algal biomass powder was used for biochemical assay.

2.3. Analysis

2.3.1. Determination of microalgal growth

Growth of the microalgal was assayed by measuring the absorption of algal culture at a specific wavelength. Sample was regularly taken to measure optical density (OD) at a wavelength of 740 nm with UV-Vis spectrometry (U-2001, Hitachi, Tokyo, Japan) [12].

2.3.2. Determination of chemical compositions of raw rice straw and rice straw hydrolysate

Glucan, xylan, lignin, protein, ash and soluble materials in raw rice straw and treated rice straw were determined according to procedures which are described in [13]. Lipid content of rice straw was determined gravimetrically after Soxhlet extraction of 300 mg the rice straw with acetone for 8 h, followed by concentrated to a dryness using a rotary evaporator [14].

Total reducing sugars were determined by the colorimetric method at 540 nm with DNS color reagents [15]. The composition of reducing sugar was characterized and quantified by a high-performance liquid chromatography coupled with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., California, USA), 5 mM sulfuric acid as eluent and UV detector (HPLC-UV Ultimate 3000, Thermo Scientific, Massachusetts, USA). Standards including glucose, xylose, arabinose, mannose and galactose were obtained from Sigma-Aldrich (Missouri, USA) [15].

The percent sugar hydrolysis efficiency (*SHE*) was determined according to following equation (1) [16]:

$$SHE = \frac{m_{TRS} \times 0.9 \times 100}{m_{cellulose+hemicellulose}} \quad (1)$$

where m_{TRS} is the weight of total reducing sugar (g), $m_{cellulose+hemicellulose}$ is weight of cellulose and hemicellulose fractions in rice straw (g).

The sugar utilization efficiency was determined according to the following equation (2).

$$H_i = \left(1 - \frac{C_i}{C_{0i}}\right) \times 100 \quad (2)$$

where H_i is the sugar utilization efficiency (%); C_{0i} is the initial concentration of sugar (g/L), C_i is the concentration of sugar measured at time slot (t) (g/L).

2.3.3. Algal biochemical assay

Major biochemical composition of the *Scenedesmus* sp. strain including lipids, carbohydrates

and proteins was also determined. Lipids were derived as fatty acid methyl esters (FAME) via *in situ* transesterification of the dry algal biomass with homogeneous catalytic solvent (HCl/methanol, 5 % v/v) at 85 °C for 1 h, followed by characterization and quantification with gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID) [17]. Total carbohydrate of the *Scenedesmus* sp.'s biomass was measured using the colorimetric method after digestion with the phenol-sulfuric acid reagent [15]. Total protein was determined using spectrophotometric assay following the Bradford procedure which was described elsewhere [15].

2.3.4. Statistical analysis

Experiments were carried out in triplicate and results were expressed as average \pm standard deviation (SD). Data was treated using Microsoft excel software 2016.

3. RESULTS AND DISCUSSION

3.1. Reducing sugar production and composition from rice straw by different treatment methods

It can be seen that the hydrolysis method affected the yield and sugar content from rice straw. The average sugar content in the acid hydrolysate was measured as 1.2 g/L which was slightly higher than 1.1 g/L determined for the alkaline hydrolysate (Figure 1A). This data agreed with results reported by Kim et al. (2022), showing that reducing sugars produced by 0.5 % H_2SO_4 was 16.8 g/L which was remarkably higher than 1 g/L determined for NaOH or NH_4OH treatment [18]. This is attributed to alkali hydrolysis can delignify lignin part [19] and cleave hydrogen bonds and covalent bonds in cellulose and hemicellulose [18], while acid hydrolysis cleaves strong chemical bonds [18]. Notably, the combined hydrolysis gave the highest sugar content of 2.2 g/L, which was two times higher than that of two separated hydrolysis methods (Figure 1A). This is reasonable because after alkaline treatment, a certain proportion of lignin was removed, exposing open structure of cellulose-hemicellulose for the acid agent to further degrade and hydrolyze for saccharides generation [20].

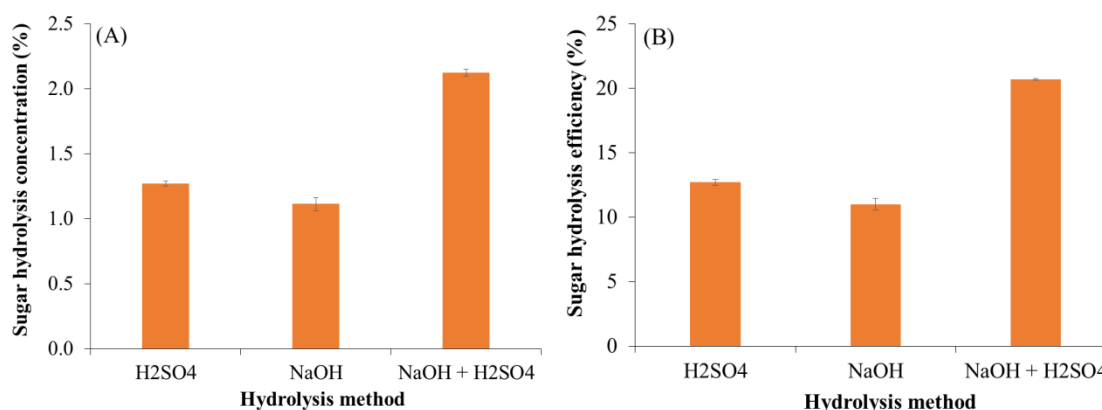


Figure 1. Total reducing sugar production (A) and hydrolysis efficiency (B) from rice straw by H_2SO_4 , NaOH and combined NaOH + H_2SO_4 methods.

Regarding hydrolysis efficiency, treatment with 10 % H_2SO_4 at 121°C for 20 minutes gave a hydrolysis efficiency of 12.7 %. Similarly, the treatment with 10 % NaOH boiled for 2 h resulted in a sugar yield of 11 %. However, the combined method using NaOH + H_2SO_4 gave the rice

straw sugar hydrolysis efficiency of up to 20.69 % (Figure 1B). The obtained data demonstrated that the combined method effectively hydrolyzed and significantly increased the amount of sugar in the hydrolysate compared to the hydrolysis method using only one agent of alkaline or acid.

It was also noted that different treatment methods resulted in different reducing sugar profiles. Data reported in Figure 2 shows that glucose, xylose and arabinose were the most abundant monosaccharides detected in rice straw hydrolysate. The concentration of glucose, xylose, and arabinose were determined in the range of 0.76 – 1.1, 0.13 – 0.76, and 0.25 – 0.27 g/L, respectively, which accounted for 51 – 74, 11 – 36 and 8 – 28 % of the total reducing sugar, respectively. Glucose concentration is the highest level due to cellulose composition in rice straw is the largest level of 43 %, following is hemicellulose of 25 %. Reducing sugar profile data displayed the linear relationship to total reducing sugar production by different hydrolytic methods.

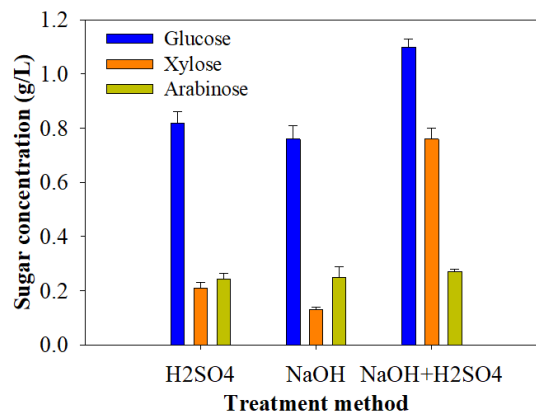


Figure 2. Monosaccharide composition of rice straw hydrolysate produced by different treatment methods (n = 3).

3.2. Growth of algal *Scenedesmus* sp. in rice straw hydrolysate

Chemical-derived hydrolysate of rice straw was a complex mixture that contained not only sugar but also inhibitors (levulinic acid, 5-HMF, and furfural) for microalgal growth [18]. Our early investigation revealed that the undiluted hydrolysate significantly inhibited the algal growth [6]. Therefore, the hydrolysate was diluted to different rates for examination of growth of *Scenedesmus* sp. Data shown in Figure 3A indicated that *Scenedesmus* sp. grew well in the acid hydrolysate at a 50 %, 25 %, 10 % dilution, and the control obtaining optical density of 0.88, 0.85, 0.46, and 0.40 Abs at day 10 of the cultivation, respectively. *Scenedesmus* sp. exhibited a lower growth in the 50%, 25 % and 10 % diluted alkaline hydrolysate, achieving optical density of 0.47, 0.49, and 0.52 Abs at day 10 of the cultivation, respectively (Figure 3B).

Notably, *Scenedesmus* sp. achieved optical density of 1.5, 0.95, and 0.52 Abs when the algal was grown in the 50 %, 25 %, and 10 % diluted hydrolysate from the combined method (Figure 3C). In all experiments, the control exhibited growth of the algal train in phototrophic mode, obtaining the adsorbance at 740 nm of around 0.41 Abs during 10 days of the cultivation (Figure 3), which was slightly lower than those achieved in the mixotrophic modes with rice straw hydrolysate supplements. The highest density of algal biomass was obtained with a 50 % dilution of the rice straw hydrolysate, which was nearly 1.6 times that of the algal biomass obtained with

25 % dilution and about 3 times that of the algal biomass obtained with a 10 % dilution rate. It was noted that 50 % dilution significantly minimized inhibiting effects of several chemicals for the development of the microalgal to obtain the optimal growth of *Scenedesmus* sp [6, 18].

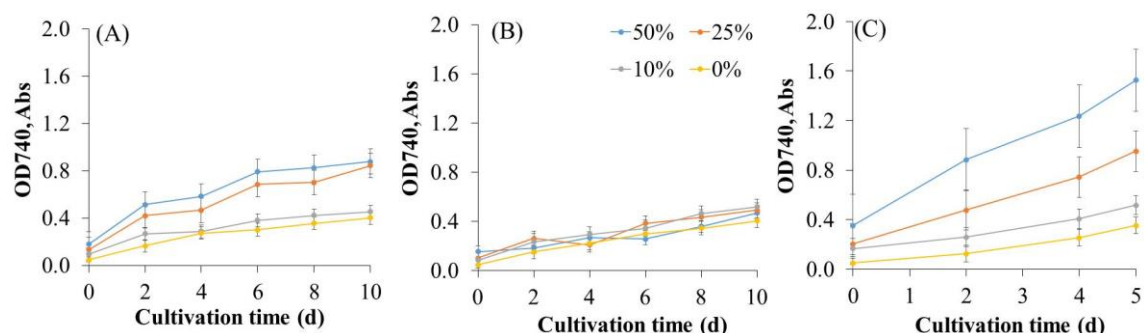


Figure 3. The growth of the algae *Scenedesmus* sp. in rice straw hydrolysate pretreated with H₂SO₄ (A), NaOH (B) and NaOH-H₂SO₄ (C).

3.3. Sugar utilization efficiency by *Scenedesmus* sp. in rice straw hydrolysis

Sugar utilization efficiency by *Scenedesmus* sp. is shown in Figure 4. Data indicates that the sugar utilization efficiency of *Scenedesmus* sp. ranged between 72 % and 87 % for the NaOH-based hydrolysate. The highest consumption of sugar was observed when 50 %- and 25 %-diluted hydrolysate were used, whereas the lower efficiency of sugar consumption was determined for 10 %-diluted hydrolysate. This was reasonable because at a lower dilution rate, a high concentration of inhibitors negatively affected the algal growth, consequently resulting in lower sugar utilization. Similarly, the sugar utilization efficiency of *Scenedesmus* sp. when it grew on H₂SO₄-based and NaOH/H₂SO₄-based hydrolysates was determined 78 – 95 % and 70 – 79 %, respectively. The lower sugar utilization efficiency in NaOH/H₂SO₄-based hydrolysates was observed because the data was measured on day 5 of the cultivation (Figure 3C), whereas data reported for H₂SO₄-based and NaOH-based hydrolysates was assayed on day 10 of the cultivation (Figure 3A-B).

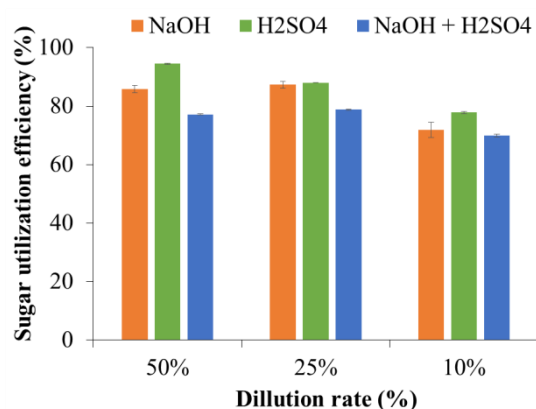


Figure 4. Sugar utilization efficiency of *Scenedesmus* sp. from rice straw hydrolysate. NaOH-treated hydrolysate (A), H₂SO₄-treated hydrolysate (B) and NaOH+H₂SO₄-treated hydrolysate (C).

This study confirms that rice straw is a rich source of carbohydrates after being chemically treated with H₂SO₄ and NaOH to cultivate the microalgae *Scenedesmus* sp. Similar to the results

obtained, the hydrolysis product from rice straw was used as a carbon source for polyunsaturated fatty acids (PUFA) production from *Chlorella pyrenoidosa* ZTY4. A sugar concentration of 10 g/L was obtained by a post-enzymatic hydrolysis process. The study showed that *Chlorella pyrenoidosa* grew well in rice straw hydrolysate, reaching biomass concentrations of 4.2 - 5.0 g/L for 6 days of the cultivation. The algal lipid content of the cell was determined up to 66.4 % [21]. El-Gamal et al. also reported a 15-day heterotrophic cultivation of microalgae *Chlorella vulgaris* and *Arthrospira platensis* on rice straw pretreated with AHP as a low-cost carbon source [22].

3.3. Biorefinery of rice straw using *Scenedesmus* sp. for circular economy development, challenges and perspectives

Major biochemical compositions of *Scenedesmus* sp. grown in different rice straw hydrolysates are reported in Table 1. Results show that protein, lipid, and carbohydrate content of *Scenedesmus* sp. biomass varied widely when the algal was grown in different hydrolysates with different dilution rates. Generally, protein, lipid and carbohydrate contents were determined as 25.7 – 45 %, 13.5 - 24.5 % and 25.4 - 42.2 %, respectively. Protein and lipids tended to accumulate higher content when *Scenedesmus* sp. grew in NaOH/H₂SO₄-based hydrolysate, whereas carbohydrates can reach a higher proportion in NaOH-based or H₂SO₄-based hydrolysate. In any case, *Scenedesmus* sp. biomass provides rich protein supplied for animal and aquafeed production [23], lipid fractionized and purified for production of nutraceuticals, food, and biofuels [24], carbohydrates hydrolyzed for chemical productions (ethanol, lactic acid, etc.) [25]

Table 1. Biochemical content of *Scenedesmus* sp. grown in different rice straw hydrolysates (n = 3).

Hydrolysate	Protein (wt.% DCW)	Lipid (wt.% DCW)	Carbohydrate (wt.% DCW)
50 % NaOH-based hydrolysate	36.6 ± 3.6	18.3 ± 0.9	27.4 ± 2.9
25 % NaOH-based hydrolysate	32.4 ± 3.1	19.6 ± 1.1	31.4 ± 3.5
10 % NaOH-based hydrolysate	29.2 ± 1.9	19.5 ± 2.5	35.2 ± 5.1
50 % H ₂ SO ₄ -based hydrolysate	25.7 ± 1.5	13.5 ± 0.6	42.2 ± 3.7
25 % H ₂ SO ₄ -based hydrolysate	37.6 ± 3.2	21.3 ± 2.4	32.7 ± 3.5
10 % H ₂ SO ₄ -based hydrolysate	35.7 ± 4.1	19.3 ± 2.1	34.6 ± 2.7
50 % NaOH+H ₂ SO ₄ -based hydrolysate	38.7 ± 2.9	22.6 ± 1.7	31.7 ± 3.7
25 % NaOH+H ₂ SO ₄ -based hydrolysate	41.2 ± 0.9	23.3 ± 2.7	30.4 ± 1.8
10 % NaOH+H ₂ SO ₄ -based hydrolysate	45.0 ± 2.5	24.5 ± 1.9	25.4 ± 4.5

DCW: Dry cell weight

The bioprocess industry based on microalgae is constantly seeking to obtain useful products from the highly abundant lignocellulosic feedstock. Although microalgae have been demonstrated to display excellent capability in the consumption of five-carbon and six-carbon sugars from lignocellulosic materials (e.g., rice straw) for the production of high-value molecules such as protein, lipid, pigments, etc, the processing of lignocelluloses into reducing sugars-rich hydrolysates is a costly process due to the strong-crystalline structure of lignocellulose materials. Chemical methods are difficult to meet the demand to completely convert lignocelluloses to sugars because of the environmental issue. Biochemical process based on enzymes is a greener alternative approach for processing lignocellulosic biomass. However,

one of the crucial challenging factors is in association with the expression levels, stability, and cost-effectiveness of the lignocellulose-degrading enzymes composed of cellulases, hemicellulases, lignases, and other accessory proteins in a non-competing, progressive, and synergetic order, in one complex. The challenge has ever been the successful assembly of an entire suite of these enzymes that could function optimally at the same time and under different conditions to completely digest lignocellulosic biomass to simple sugars. Many cellulase improvement practices and enzyme systems (i.e., cell-free or whole-cell) have surfaced and presently the fraternity is witnessing a gradual shift towards the cell-surface display system. Nevertheless, the remaining challenge was the achievement of high-level expressions necessary for industrial use at low cost for sugar production. To further improve lignocellulose activity, techniques such as directed evolution and rational design have ever been used for harnessing glycosylation in a 'green' approach. The success of the approach is a promising application of consolidated bioprocessing of lignocelluloses to reducing sugars, which are substrates for microalgae production.

4. CONCLUSIONS

Pretreatments of rice straw with NaOH, H₂SO₄, and NaOH+H₂SO₄ were studied, achieving hydrolysates with reducing sugar concentrations of 1.1, 1.2, and 2.2 g/L, and equivalent sugar hydrolysis efficiencies of 11, 12.7, and 20.7 %, respectively. The *Scenedesmus* sp. strain was successfully grown in rice straw hydrolysates derived from NaOH, H₂SO₄, and NaOH+H₂SO₄ treatments. The optimal growth of *Scenedesmus* sp. was recorded in the hydrolysate with 50 % dilution, achieving biomass density of 0.52 - 1.5 Abs with sugar utilization efficiency of 70 - 94 %. In addition, the harvested *Scenedesmus* sp. biomass comprised of protein, lipid and carbohydrate contents were determined as 25.7 - 45 %, 13.5 - 24.5 %, and 25.4 - 42.2 %, respectively. Biorefinery of rice straw using bioprocess of microalgae can create a circular economy with low-carbon emission. Despite of its promising, processing of lignocelluloses into reducing sugars-rich hydrolysates for microalgae production is very challenging. The future work is further application of enzymatic hydrolysis in a consolidated bioprocessing for chemical-treated rice straw, expecting to produce high concentration of reducing sugars with 100 % degradation efficiency to enhance biomass production from *Scenedesmus* sp.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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