Biorefining of corn stover for efficient production of bioethanol, biodiesel, biomethane, and value-added byproducts

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Abstract

The present study investigated an integrated biorefinery that employed corn stover as the feedstock for sustainable bioethanol, biodiesel, biogas, chitosan, glycerol, and animal feed production. Corn stover was initially subjected to dilute acid pretreatment (1.8 % v/v H_2SO_4, 121°C, and 22 min) followed by enzymatic hydrolysis with a commercial cellulase (37°C, 72 h) to promote the release of glucose (~93wt.%) and xylose (~89 wt.%). *Mucor indicus* fungus was then employed to convert the released sugars into bioethanol, glycerol, and fungal biomass with yields of 0.38 g g^{-1}, 36 mg g^{-1}, and 0.51 g g^{-1}, respectively. The biomass of *M. indicus* was processed to extract chitosan (6 mg g^{-1} fungal biomass) and lipids (297 mg g^{-1} fungal biomass). The lipid was subsequently converted to biodiesel via transesterification in the presence of HCl/ MeOH with the yield of 0.54 g g^{-1} fungal lipid. The defatted biomass residue was then converted to biogas with 81 % theoretical yield through anaerobic digestion.. To ensure process circularity, the nutritional values of pretreated and hydrolyzed corn stover were also investigated with their suitability as livestock. Based on this study, it was determined that 158.1 thousand tons of dry corn stover, which was annually available for collection in Iran, could be used for the production of 137.6 kg chitosan, 10.4 ton animal feed, 870.0 kg glycerol, 40.7 million litters ethanol, 2.8 million m^3 biodiesel, and 449.2 million m^3 biomethane. The utilization of produced ethanol, biodiesel, and biomethane in transporting sector was shown to have the potential of facilitating 4.3 million tons of equivalent carbon dioxide and a 197.8 million dollars reduction of associated social costs.

Keywords: Corn stover, Biorefinery, Biofuels, value-added byproducts, *Mucor indicus*, Socioeconomic assessment

1. Introduction

The global climate has been changing, leading to negative impacts on the environment, human health, and worldwide economy, with the main culprit of this climate change, determined to be high levels of greenhouse gases (GHGs) such as SOx, NOx, and CO_2 [1, 2]. These GHGs are released into the atmosphere when fossil fuels are transformed into energy via combustion [1]. Thus, replacing fossil fuels with biofuels has been proposed as constituting a viable pathway to reduce greenhouse gas emissions, since biofuels are recognized as carbon neutral [3, 4]. Indeed, according to the International Energy Agency (IEA), the transition of at least a quarter of the world's transportation fuels to biofuels by 2050 is necessary to mitigate future climate catastrophes. The IEA also encourages the production of biofuels from non-food-crop feedstocks such as wastes and residues [5]. This is because, in addition to these feedstocks not being edible, their valorization to produce valuable products will facilitate the resolution of their associated pollution challenge, leading to enhanced environmental sustainability outcomes [6, 7]. In particular, agricultural residues (e.g., corn stover, rice straw, and wheat straw) have a potential for biofuel production due to their favorable availability, lower price, and renewability properties [8, 9].

Corn stover constitutes an abundant agricultural residue that could potentially be used for biofuel production [10]. Annually, over one billion tons of corn stover is produced globally, with a harvest index ranging from 47 % to 56 % [11]. After harvesting, the remaining corn stover on the farm is typically managed via its addition to the soil to improve its fertility or burned to annihilate diseases and pests in the agricultural land [12]. By considering these agricultural protection aspects, Karimi et al. [13] proposed the collectible amount of corn stover residues that could be utilized as the feedstock for biofuel production. Indeed, in the European Union and the United States, the

conversion of corn stover residues to biofuels has been estimated to prevent the emission of 18 million tons of CO_2 that may be generated from its direct combustion [3].

The lignocellulosic structure of corn stover is composed of cellulose (32-36 %), hemicellulose (18-21 %), and lignin (11-14 %) [14]. To produce biofuels from this recalcitrant structure, physical [15, 16], chemical [17, 18], or biological [19, 20] pretreatment is essential [21]. Among the different processing methods, the pretreatment using dilute sulfuric acid has been identified as effective since the approach promotes the efficiency of subsequent saccharification and hemicellulose solubilizing processes [22, 23]. The commercial application of this approach may however be limited by the associated high costs of acid recovery and pretreatment facilities [24]. This limitation can be mitigated by applying the biorefinery approach in the processing of corn stover since the efficiency of biofuel production can be enhanced while simultaneously producing additional high-value products that could serve as additional revenue streams [3, 25-27]. Notably, minimizing waste generation and improving the economy of the process by producing multi-high-value products are among the main advantages of biorefineries [21]. An example is corn stover-ethanol biorefinery with value-added byproducts such as acetic acid, phenol, furfural, cresols, catechol, formic acid, and acetaldehyde [28]. Besides, furan-based biofuels, specifically dimethylfuran, have been currently considered as a target product of lignocellulosic biorefinery [29-31]. Therefore, commercializing biofuel production from corn stover significantly depends on the generation of additional value-added byproducts in the biorefinery [3].

To this regard, the present study sought to develop a Phase II biorefinery [32] that can convert corn stover to multiple valuable products such as biofuels (bioethanol, biodiesel, and biogas) and valuable byproducts (animal feed, glycerol, and chitosan). To demonstrate the sustainability of the developed biorefinery, a socioeconomic analysis was undertaken to estimate its greenhouse gas

emissions (GHG) reduction potential and the associated social cost of carbon dioxide (SCC), based on existing information regarding the biofuels that can potentially be produced from corn stover in Iran. To the best of our knowledge such a comprehensive study, investigating the integration of several corn stover conversion technologies and unit operations to produce high-value products, as a basis of conducting rigorous socioeconomic analysis, is yet to be undertaken in the literature. This novel study, therefore, sought to bridge this knowledge gap.

2. Materials and Methods

The experiments in this study included feedstock preparation, pretreatment, hydrolysis, byproduct extraction, and biofuel production. This section consists of an explanation of the materials and methods used in each part.

2.1. Feedstock preparation

Corn stover samples were collected from corn stover residues left on farms located in Alavijeh, Isfahan, Iran (33° 3′ 10″ N, 51° 4′ 57″ E). The samples were air dried at ambient temperature to achieve constant weight, then milled and screened, and particles with the size of less than 1 mm were collected. Afterward, the samples were placed in an air-tight plastic bag and stored at room temperature until further use.

2.2. Feedstock characteristics

The standard method of determination of structural carbohydrates and lignin provided by the National Renewable Energy Laboratory was applied to characterize the lignocellulosic composition of corn stover [33]. Accordingly, 30 mg corn stover was mixed with 3 mL of 72 % (w/w) sulfuric acid in a 100 mL glass bottle and placed in a water bath at 30 °C for 1 h. Afterward, 84 mL of deionized water was added such that the sulfuric acid concentration in samples was

adjusted to 4 % (w/w). The samples were then put in an autoclave at 121°C for 1h to complete the dilute acid hydrolysis. Finally, the monomeric sugars, e.g., glucose and xylose, that were released in the liquid phase were analyzed using high-performance chromatography (HPLC). Also, the lignin content was determined as the difference between the weight of hydrolyzed biomass before and after burning at 575 °C for 24 ± 6 h.

2.3. Pretreatment

Due to the recalcitrant structure of untreated corn stover, its enzymatic hydrolysis would be inefficient and slow. To increase the rate and efficiency of future enzymatic hydrolysis, a pretreatment step using dilute acid treatment was employed prior to ethanol production [34]. Dilute acid treatment was selected due to its capacity to facilitate an efficient separation of the polysaccharides of cellulose and hemicellulose [35]. To this regard, the optimum conditions for dilute acid treatment (1.8 % v/v H_2SO_4, 121 °C, and 22 min), obtained in our previous work, were used to undertake the dilute acid pretreatment of corn stover, at a mass concentration of 10 % dry matter [36], in an autoclave. Once the samples were cooled to room temperature, the resulting solid (S_a) and liquid (L_a) phases were separated using a filter paper (Whatman paper no. 40). Before applying the pretreated solid (S_a) at enzymatic hydrolysis, it was washed with distilled water. Washing with water is suggested to remove the produced/released inhibitors and neutralize the pretreated solid [37]. Despite the generation of wastewater, washing with water can prevent salt formation compared to naturalization with base. Thus, the S_a was initially washed with distilled water until the pH of the wash water was 4.5-5.5 and subsequently air dried to constant mass overnight. . The recovered L_a phase was collected and stored in a freezer at -20 °C until further use.

2.4. Enzymatic hydrolysis

The enzymatic hydrolysis of pretreated corn stover (i.e., S_a) was performed in batch mode, using a 118 mL glass bottle. Also, corn stover without pretreatment was hydrolyzed to assess the effect of pretreatment on monosaccharide release. The hydrolysis medium was prepared with 8 % (w/w) of S_a in buffer citrate (pH 4.8) and autoclaved at 121 °C for 20 min. Once the slurry cooled to room temperature, 15 FPU g^{-1} cellulase enzyme (Celluclast 1.5 L, Novozymes, Denmark) was loaded in the bottles. The activity of cellulase was determined to be 49 FPU mL^{-1} using the Andey and Baker method [38]. Afterward, the samples were placed in an incubator at 37°C for 72 h to complete enzymatic hydrolysis. The liquid (L_b) phase was then separated/recovered and stored at -20 °C until further use. Eq. 1 [39] was applied to calculate the enzymatic hydrolysis yield as follows:

$$\text{The yield of enzymatic hydrolysis } (\%) = \frac{Concentration\ of\ produced\ glucose\ (g\ L^{-1})}{1.111 \times glucan\ in\ substrate\ (g\ L^{-1})} \times 100 \quad \text{Eq. 1}$$

where 1.111 is the glucan hydration factor.

The solid (S_b) phase was also washed with distilled water, air-dried, placed in an air-tight plastic bag, and kept at room temperature until further analysis.

2.5. Characterizing pretreated and enzymatically hydrolyzed corn stover as animal feed

Some essential parameters for animal feed were measured in the case of pretreated and hydrolyzed corn stover (i.e. S_b) and compared to untreated corn stover as a traditional animal feed. In this regard, the dry matter was determined after drying at 105 °C, according to AOAC official method 930.15 [40], and ash content was measured after burning at 550 °C, according to AOAC official method 942.05 [41]. Crude fat was also defined as extracted ones with petroleum ether for 6 hours

(AOAC official method 920.39 [42]). Crude protein (CP) was calculated using the Kjeldahl procedure, which involves acid digestion and distillation, with 6.25 employed as the conversion factor [43]. Acid detergent fiber (ADF), non-fibrous carbohydrates (NFC), neutral detergent fiber (NDF), and acid detergent lignin (ADL) were measured according to the methods of Van Soest et al. [44]. Neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP) were analyzed using the Cornell Net Carbohydrate and Protein (CNCP) method [45]. Total digestible nutrient (TDN), digestible energy (DE), metabolizable energy (ME), and net energy lactation (NEL_{3x}) were calculated according to equations presented by National Research Council (NRC 2001) [46].

2.6. Microorganism and fungal biomass production

The zygomycete fungus *M. indicus* CCUG 22424 (The Culture Collection of the University of Gothenburg, Sweden) was used in the experiments. *M. indicus* is a zygomycete fungus that was selected due to its reported favorable performance in ethanol production from xylose, glucose, and lignocellulosic hydrolysate [47]. Additionally, *M. indicus* has the potential to produce substantial masses of fungi biomass that contains high concentrations of fatty acids and chitosan [48-50]. To this regard, *M. indicus* was incubated in an agar slant containing: 20 g L^{-1} agar, 40 g L^{-1} D-glucose, and 10 g L^{-1} peptone, at pH 5.5 and 32 ° C for five days to form spores on the plates. Afterward, to obtain a cell density of 3 g dry weight L^{-1}, the spores (concentration of $6 \pm 3 \times 10^6$ spores mL^{-1}) were washed, suspended in sterilized distilled water, and cultivated in a solution containing: 5 g L^{-1} yeast extract, 7.5 g L^{-1} $(NH_4)_2SO_4$, 3.5 g L^{-1} KH_2PO_4, $MgSO_4.7H_2O$, 0.75 g L^{-1} 1.0 g L^{-1} $CaCl_2$, and 50 g L^{-1} glucose at 32 °C and 150 rpm for 24 h. The resulting fungal biomass was collected via centrifugation at 4000 rpm for 10 min and washed twice with distilled water.

2.7. Aerobic/Anaerobic cultivation

Anaerobic cultivations were performed in 118 mL serum glass bottles, and aerobic cultivations were conducted in 200 mL Erlenmeyer flasks. The carbon source for anaerobic cultivation was supplied from the liquid fraction of enzymatic hydrolysis step (i.e. L_b) while the dilute acid hydrolysate (i.e. L_a) was employed as the carbon source for aerobic cultivation and supplemented with required nutrients, according to the methodology presented by Karimi et al. [51]. A reference media composed of pure glucose in anaerobic cultivation was prepared at a similar glucose concentration in the enzymatic hydrolysate. Also, a solution of glucose and xylose at similar concentrations to those in the dilute acid hydrolysate was prepared and considered as the reference media in aerobic cultivation. The pH of the cultivation media was adjusted to 5.5 and then autoclaved at 121 °C for 10 min. Anaerobic cultivations were inoculated with the biomass of *M. indicus* while imposing a biomass concentration of 3 g dry weight L^{-1}. The bottles were closed tightly using butyl rubber septum and aluminum caps and purged with pure nitrogen to establish the anaerobic condition. The bottles were then placed in an incubator at 32 °C and 120 rpm. After 48 h, the liquid (L_c) and solid (S_c) phases were separated by centrifuge (10 min, 4000rpm) under sterile conditions. The L_c phase, containing mainly ethanol, was kept at -20 °C until further analysis. The S_c phase containing mainly *M. indicus* cells was inoculated to the Erlenmeyer to continue their growth aerobically in an incubator at 32 °C and 120 rpm for 72 h. At the end of aerobic cultivation, *M. indicus* cells were recovered using a filter paper, then washed thrice with distilled water prior to freeze drying. The freeze-dried biomass of *M. indicus* was subjected to lipid and chitosan extraction. Also, the filtrate was kept for analysis of possible ethanol and glycerol contents. All of the experiments were carried out in duplicate.

The yield of ethanol in aerobic/anaerobic cultivations was calculated using Eq.2 [39].

$$Ethanol\ yield\ (g\ g^{-1}) = \frac{Produced\ ethanol\ (g\ L^{-1})}{1.111 \times 0.51 \times glucose\ concentration\ (g\ L^{-1})} \quad \text{Eq. 2}$$

where 1.111 is the glucan hydration factor and 0.51 is the theoretical yield of ethanol from hexoses. Moreover, the yield of glycerol was calculated using the following equation (Eq. 3):

$$Glycerol\ yield\ (g\ g^{-1}) = \frac{Glycerol\ concentration\ (g\ L^{-1})}{Glucose\ concentration\ (g\ L^{-1})} \quad \text{Eq. 3}$$

2.8. Determination and extraction of lipid

The lipid content of *M. indicus* cells was extracted using the Blight and Dyer Method [52]. This method used chloroform: methanol: water with a volumetric optimum ratio of 2: 2: 1 to enable the extraction of intracellular lipids from fungal biomass. The amount of extracted lipid was expressed as a gram per gram of dry fungal biomass.

2.9. Determination and extraction of chitosan

In order to extract chitosan from the fungal cells, it is necessary to eliminate protein from the cells. Thus, 1 gram of dried fungal biomass was suspended in 30 mL of 0.5 M NaOH and autoclaved for 20 min at 121 °C. Then the alkali-insoluble materials (AIM) were subsequently separated using a centrifuge (4000g, 10 min) and washed several times with distilled water. The AIM was then freeze-dried and weighed. The subsequent extraction of chitosan from AIM was performed according to the method developed by Naghdi et al. [53]. Briefly, 0.25 g of AIM was suspended in 25 mL of 0.1 N H_2SO_4 and stirred at room temperature for 30 min. After re-centrifugation, the solid phase was separated and washed three times using distilled water. This step was followed by the addition of 0.1 % (w/v) NaOH followed by the washing using distilled water. In the end, the

sample was treated with 25 mL of 0.1 N lactic acid at room temperature while stirred simultaneously (150 rpm, 1 h). The solution was centrifuged (4000×g, 10 min), the supernatant was separated, and its pH was adjusted to 10 by 2 M NaOH. At this time, chitosan was precipitated with the sediment carefully collected and washed several times with distilled water. The recovered chitosan was freeze-dried, weighted, and characterized by comparing its spectrum to commercially sourced authentic chitosan standard, using FTIR. The yield of chitosan, as one of the final products of the proposed biorefinery, was calculated according to the following equation (Eq.4):

$$Chitosan\ yield\ (g\ g^{-1} dry\ fungal\ biomass) = Chitosan\ yield\ (g\ g^{-1} AIM) \times AIM\ yield\ (g\ g^{-1} dry\ fungal\ biomass) \quad \text{Eq. 4}$$

The determination of N-acetyl glucose amine, glucose amine, and phosphate contents in the fungal cell walls was performed according to the method described by Mohammadi et al. [54]. Also, Eq. 5 was used to calculate the degree of deacetylation (DD) of purified chitosan [55].

$$\mathrm{DD} = \left(1 - \frac{GlcNAc}{GlcN}\right) \times 100 \quad \text{Eq. 5}$$

2.7. Biodiesel production

Transesterification of lipids extracted from *M. indicus* cells was conducted according to the method presented by Laurens et al.[56]. Briefly, 1.0 g fungal lipid was subjected to 60 mL of 5 % v/v HCl in methanol at 85 °C. Hydrochloric acid catalyzed the transesterification reaction, and fungal lipids and methanol were the reactants. After one hour, the reaction products were separated into two phases using a centrifuge (4000 rpm, 15 min). Then the upper phase was precisely drained and weighted to determine the biodiesel yield according to Eq. 6 [57].

$$Conversion\ yield\ of\ lipid\ to\ biodiesel\ (\%) = \frac{Produced\ biodiesel\ (g)}{Lipid\ weight\ (g)} \times 100 \quad \text{Eq. 6}$$

2.9. Biogas production

The cell residues after lipid extraction were applied to biogas production according to the method presented by Hensen et al. [58]. Also, the whole cells of *M. indicus* were subjected to mesophilic anaerobic digestion to determine their biogas production potential. Briefly, the amount of 0.25 g cells of defatted cell residues and 20 mL inoculum provided from a 7000 m^3 digester (Isfahan Municipal Wastewater Treatment, Isfahan, Iran) were mixed with 5 mL deionized water in a 118 mL dark bottle. The bottles were then sealed using rubber septum and aluminum caps and purged with pure nitrogen to establish the anaerobic condition. Afterward, the bottles were placed in an incubator at 37 °C for 40 days and shaken manually once a day. Every 3 days, samples were recovered, and the compositional distribution of the biogas was subsequently analyzed using gas chromatography. For calculating the amount of produced methane, as one of the target products of the biorefinery, Eq. 7 was applied:

$$Methane\ yield\ (mL\ g^{-1} dry\ biomass) = Methane\ yield\ (ml\ g^{-1} VS) \times VS\ (g\ VS\ g^{-1}\ dry\ biomass) \quad \text{Eq. 7}$$

where VS (volatile solids) was measured for fungal biomass according to the standard method provided by the National Renewable Energy Laboratory [33].

2.10. Analytical method

A high-performance liquid chromatography (HPLC, Agilent 1100, Agilent Technologies, CA, USA) equipped with a refractive index (RI) detector and a Bio-Rad Aminex HPX-87P analytical column (Bio-Rad, CA, USA) was used to quantify the sugars. The eluent was deionized water at 85 °C with a flow rate of 0.6 mL min^{-1}. Also, ethanol was determined using the same HPLC device, equipped with an ion exchange Aminex column (HPX-87H, Bio-Rad, CA, USA). The mobile phase was 5 mM H_2SO_4 at 60 °C with a flow rate of 0.6 mL min^{-1}.

To analyze GlcN and GlcNAc contents of AIM, an HPLC device (Jasco International Co., Tokyo, Japan) equipped with ion-exchange Aminex column (HPX-87H, Bio-Rad, Richmond, CA) at 60 °C with 0.6 mL min^{-1} eluents of 5 mM H_2SO_4 as mobile phase was used. The determination of 2,5-anhydromannose and acetic acid was performed using a RI and UV-vis detector, respectively. Fatty acid composition of fungal lipid was determined using gas chromatography (Sp3420A, Beijing Beifen Ruili Analytical Instrument Co., China) equipped with a capillary split less injection, a flame ionization detector (FID), and a SolGel-WAX column (30 m × 0.25 mm internal diameter × 1.0 mm film, SGE Analytical Science Pty Ltd., Ringwood, Australia). Nitrogen with a flow rate of 1.0 mL min^{-1} was used as carrier gas. The injector temperature, flame ionization detector temperature, split flow, and split ratio were adjusted at 220 °C, 250 °C, 1.6 mL min^{-1}, and 20:1, respectively. Furthermore, the content of fatty acid methyl esters (FAME) was determined using the method developed by Sabzalian et al. [59]. The retention time of each fatty acid was compared with fatty acid standards (C14:0 to C24:1) for identification.

2.11. Mass balance

The analysis of mass balance was performed according to the data obtained from dilute-acid pretreatment, enzymatic hydrolyses, fermentation, transesterification, and anaerobic digestion. The yields of products and byproducts, including chitosan, animal feed, glycerol, bioethanol, biodiesel, and biomethane, were calculated for 1 kg of dry corn stover as the biorefinery feedstock. The calculation of mass yields (X_i) was performed according to Eq. 8:

$$X_i(g) = Y_i \times m_i \quad \text{Eq. 8}$$

where m_i(g) refers to the weight of glucose, xylose, or fungal biomass that resulted in forming the corresponded products, i.e., ethanol, glycerol, chitosan, biodiesel, and biomethane, and Y_i refers to the yield of fermentation, digestion, or extraction.

2.12. Socioeconomic analysis

The positive impacts of biofuel production in this biorefinery on reducing greenhouse gas emissions (GHG) and connected social cost (SCC) were quantified using equations 9-20.

Firstly, the collectible amount of corn stover for biofuel production was measured as follows [13]:

$$Q_R = (1 - f_m) \times Q_{R,W} \quad \text{Eq. 9}$$

$$Q_{R,W} = f_{CR}(P_{Corn} \times RPR - S_{Corn} \times f_{GC}) \quad \text{Eq. 10}$$

where $RPR, P_{Corn}, Y_{Corn}, S_{Corn,} Q_{R,W}$, and Q_R are the amounts of residue to product ratio, corn stover production (t), corn stover yield (t ha^{-1}), area harvested (ha), collectible wet residues (t), and collectible dry residues (t), respectively. Also, f_{CR}, f_{GC}, and f_m denote residue collectible efficiency, the factor of ground cover (t ha^{-1}), and moisture content by weight, respectively.

The quantity of each biofuel ($Q_{biofuel}$(L)) that could be produced annually from collectible corn stover residues through the proposed biorefinery was calculated by Eq. 11 [13]:

$$Q_{biofuel} = Y_{biofuel} \times Q_R \quad \text{Eq. 11}$$

where $Y_{biofuel}$ is the combined yield of bioethanol, biodiesel, and biogas (L t^{-1} dry corn stover).

Bioethanol is blended with gasoline and biodiesel with diesel as a transportation fuel. Also, biogas, after refining, would be replaced with compressed natural gas (CNG) in the transport sector. The total amount of fossil-bio fuel blend (Q_{blend}(L)) was calculated by Eq. 12. The amount of *x* in this equation denotes the volume fraction of biofuel in the fossil-bio fuel blend. In addition, the amount of saved fossil fuel (Q_{saving}(L)) due to using a biofuel blend was computed using Eq. 13. To estimate the amount of fossil fuel saving or equivalent to the blend (Eq. 14), the parameter of energy ratio (R) was used and computed by dividing the energy density of biofuels into fossil fuels.

$$Q_{blend} = \frac{Q_{biofuel}}{x} \quad \text{Eq. 12}$$

$$Q_{saving} = R \times Q_{biofuel} \quad \text{Eq. 13}$$

$$Q_{equivalent} = Q_{saving} + (1 - x)Q_{blend}$$ Eq. 14

For determination of the reduction in GHG emissions, well-to-wheel GHG emission factors are used as follows [60]:

$$\Delta_{GHG} = C_{fossil}Q_{equivalent} - C_{blend}Q_{blend}$$ Eq. 15

$$C_{blend} = xC_{biofuel} + (1 - x)C_{fossil}$$ Eq. 16

where C_{fossil}, $C_{biofuel}$, and C_{blend} indicate the well-to-wheel GHG emissions of fossil fuel, biofuel, and the blend (t CO_2 eq l^{-1} fuel), respectively.

The reduction in the total SCC (Δ_{SCC} (\$)) resulting from substituting biofuels with fossil fuels is calculated by Eq. (17) [60]:

$$\Delta_{SCC} = \Delta_{GHG} \times SCC$$ Eq. 17

The SCC is estimated to be 40 \$ to 220 \$ for each tone of emitted carbon dioxide. However, according to the United States Environmental Protection Agency (EPA) information, the SCC can be estimated for a given year by the following equation [61]:

$$SCC = 1.0286 \times Y - 2031.8$$ Eq. 18

where Y refers to the year, 1.0286 and 2031.8 are constants with units of \$ $year^{-1}$ and \$, respectively.

3. Results and discussion

The results obtained in this work were divided into different parts, i.e., dilute-acid pretreatment, enzymatic hydrolysis, byproduct extraction, and biofuel production. This section presents and discusses the results obtained in each part.

3.1. Dilute acid pretreatment

The compositional analysis of untreated corn stover showed that the glucan content was 49.3 wt.%, xylan 11.2 wt.%, and acid-insoluble lignin 12.8 wt.%. Having concluded the pretreatment, in

accordance to methods presented in section 2.3 above, glucose and xylose were produced at the yields of 54 wt.% and 89 wt. %, respectively as shown in Figure 1. Notably, the concentration of hydroxyl-methyl furfural (HMF) and furfural were 0.02 and 0.002 g g^{-1} dry corn stover, respectively, reinforcing the suitability of the dilute acid pretreatment approach for processing corn stover. The compositional analysis of pretreated corn stover showed that glucan was the dominant carbohydrate (89.9 wt.%), followed by lignin (20.3 wt.%) (Figure 1). The xylan content (1.2 wt.%) showed that it has been almost released into the liquid phase in the form of xylose and furfural. Also, 29 % of the initial mass of biomass was recovered after pretreatment, as the solid fraction which is composed of lignin, ash, and residual polysaccharides. These results are comparable with the previous studies by Zhang et al. [62] on dilute acid hydrolysate of corn stover. They reported the yield of 84.5 % and 49.7 % for the releasing of glucose and xylose, respectively, from corn stover pretreated with 4 % w/w H_2SO_4 at 190 °C for 3 min. Moreover, the higher yield of xylose recovery (86 %) was obtained after the pretreatment with 1.2% w/w H_2SO_4 at 160 °C for 8 min [63]. Generally, more severe pretreatment resulted in more hemicellulose and cellulose solubility [64]. The difference in corn stover type, temperature, and reactor types could be the reason for differences in glucose and xylose yields of this study with previously reported results.

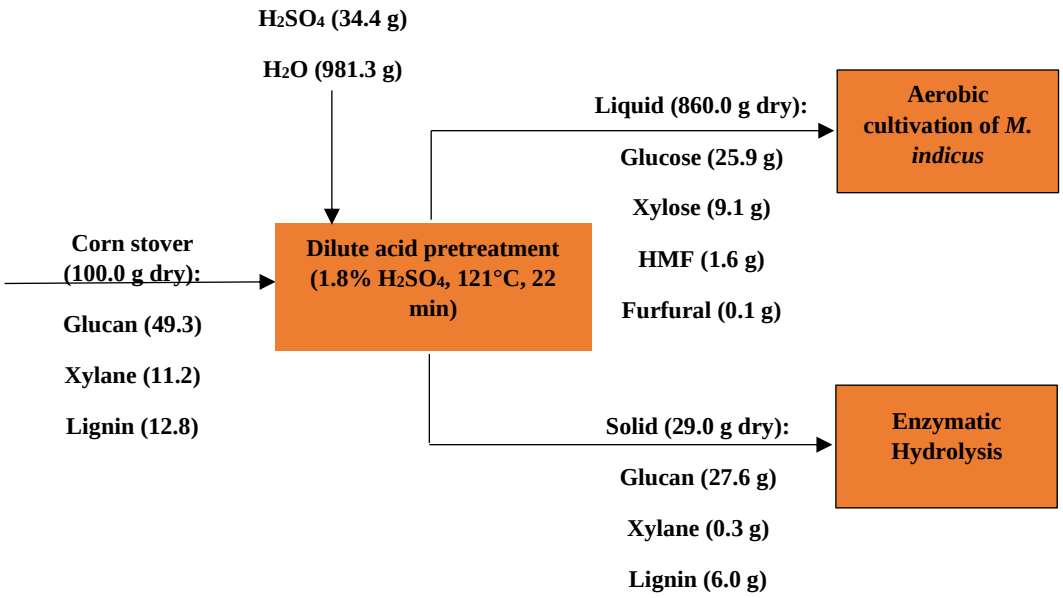

Fig. 1. Composition of corn stover before and after diluted acid pretreatment. All the experiments were performed in duplicate, and the average standard deviation was less than 0.3 % for glucan, 0.1 % for xylan, 0.4 % for lignin, 0.4 g L^{-1} for glucose, 0.2 g L^{-1} for xylose and HMF, and 0.01 g L^{-1} for furfural.

3.2. Enzymatic hydrolysis and anaerobic cultivation

The enzymatic hydrolysis of pretreated corn stover, mainly composed of glucan, was complemented using cellulase. After 72 h of enzymatic hydrolysis, 67.2 g L^{-1} and 10.8 g L^{-1} of glucose were released in the liquid phase from the pretreated and the untreated corn stover respectively. This result showed that, as expected, the dilute acid pretreatment enhanced glucose release during the enzymatic hydrolysis process. The fermentation of the liquid phase under the action of *M. indicus*, under anaerobic conditions, was then undertaken and compared to the synthetic media. The results of glucose consumption and ethanol production during fermentation are presented in Figure 2. The results show that the glucose was assimilated in less than 24 h, with

maximum ethanol yields of 0.38 g g^{-1} (76.2 wt.%) for enzymatic hydrolysate of corn stover and 0.45 g g^{-1} (88.7 wt.%) for synthetic media obtained. Also, the maximum glycerol yield was 0.01 g g^{-1} for enzymatic hydrolysate of corn stover and 0.07 g g^{-1} for synthetic media (data not shown). These results are consistent with the results presented by Karimi et al. [65], where a lower ethanol yield from the hydrolysate of rice straw compared to synthetic media (i.e., 0.35 g g^{-1} vs. 0.46 g g^{-1}) was obtained in anaerobic cultivation of *M. indicus*. Furthermore, Figure 2 shows that glucose in the enzymatic hydrolysate was not entirely assimilated by *M. indicus* cells, while no glucose was detected in synthetic media after 24 hours. Other works have also observed this phenomenon [66-68], which is hypothesized to be due to the hydrolysis of microorganism cell walls by the remaining enzyme in enzymatic hydrolysate as well as the production of reducing sugars [68].

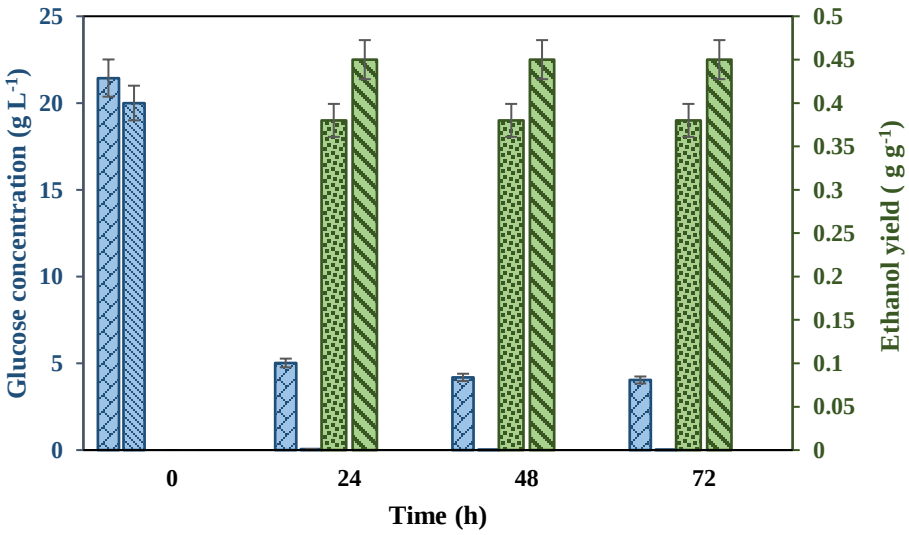

Fig. 2. Ethanol yield (g g^{-1}) and glucose concentration (g L^{-1}) during anaerobic cultivation of *M. indicus* in synthetic media and enzymatic hydrolysate of corn stover. The symbols represent the: concentration of glucose in synthetic media (▨), the concentration of glucose in the enzymatic hydrolysate of corn stover (▨), ethanol yield in cultivation on synthetic media (▩), and ethanol yield in cultivation on enzymatic hydrolysate of corn stover (▩).

3.3. Aerobic cultivation

The dilute acid hydrolysate was used as the carbon source for aerobic cultivation. Figure 3 shows the concentrations of glucose and xylose during this aerobic cultivation. The *M. indicus* cells assimilated glucose within the first 24 h and then consumed xylose within the next 48 h. Also, the yields of ethanol and glycerol are presented in Figure 4. The ethanol yield was observed to increase to 0.45 g g^{-1} within the first 24 hours and then decreased to 0.38 g g^{-1} over the following 48 hours. The maximum glycerol yield was 26.6 mg g^{-1} and 36.0 mg g^{-1} after 24 h for synthetic media and dilute acid hydrolysate of corn stover, respectively. These results are consistent with the results obtained in the aerobic cultivation of *M. indicus* by other researchers [36, 50, 51]. However, in contrast to anaerobic cultivations, the ethanol yield in the presence of dilute acid hydrolysate was 1.5 times more than in synthetic media, which is inline of the results presented by Lenartson et al. [69]. It is related to the presence of major inhibitors, i.e., HMF, furfural, and acetic acid, which are produced/released during dilute acid hydrolysis, that induce *M. indicus* cells to produce more ethanol instead of increasing their biomass [69].

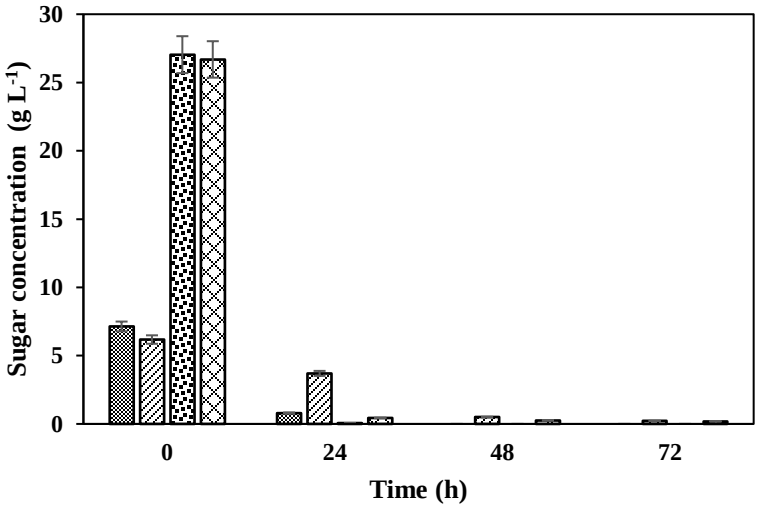

Fig. 3. Sugar concentration (g L^{-1}) during aerobic cultivation of *M. indicus* in synthetic media and dilute acid hydrolysate of corn stover. The symbols represent the: concentration of xylose in synthetic media (▩), the concentration of xylose in the dilute acid hydrolysate of corn stover (▨), the concentration of glucose in synthetic media (⊡), and the concentration of xylose in the dilute acid hydrolysate of corn stover (⊠)

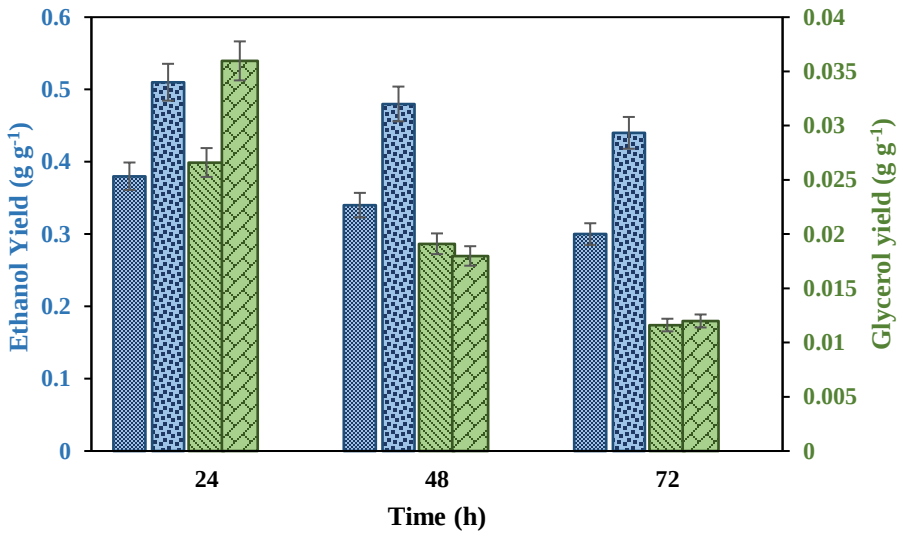

Fig. 4. Ethanol yield (g g^{-1}) and glycerol yield (g g^{-1}) during aerobic cultivation of *M. indicus* in synthetic media and dilute acid hydrolysate of corn stover. The symbols represent the: ethanol yield in synthetic media (▩), ethanol yield in the dilute acid hydrolysate of corn stover (⊡), glycerol yield in cultivation on synthetic media (▧), and glycerol yield in cultivation on dilute acid hydrolysate of corn stover (⊠).

3.4. Biomass and lipid production in *M. indicus* cells

The yields of produced biomass and extracted lipids of *M. indicus* cells during anaerobic and aerobic cultivation are presented in Table 1. In anaerobic cultivation, the biomass and lipid yields were 0.15 g g^{-1} and 30.60 mg g^{-1}. These yields increased to 0.51 g g^{-1} and 151.30 mg g^{-1} in aerobic cultivation. These amounts met the results previously reported [48, 50] on the evaluation of biomass and lipid yield of *M. indicus* in different culture conditions. When grown in synthetic

media, the biomass and lipid yields were slightly higher than cultivated in dilute acid hydrolysate, either aerobically or anaerobically. Table 1 also shows that the lipid contents were comparable for different aeration conditions and carbon sources considered in the study.

Table 1. The yield of produced biomass (g g^{-1} carbon source) and extracted lipid (mg g^{-1} carbon source), as well as lipid content (% wt) of *M. indicus* cells cultivated in aerobic and anaerobic cultivation

Aeration conditions	Carbon source	Biomass yield (g g^{-1})	Lipid content (% wt)	Lipid yield (mg g^{-1})
Anaerobic	Glucose	0.15±0.01	20.40±0.70	30.60±0.31
Aerobic	Glucose and xylose	0.55±0.02	19.50±0.20	156.91±2.11
Anaerobic	Enzymatic hydrolysate	0.14±0.01	18.90±0.90	27.72±0.23
Aerobic	Dilute-acid hydrolysate	0.51±0.03	19.10±0.20	151.30±2.00

3.4. Lipid extraction and biodiesel production

The fatty acid composition of lipids extracted from *M. indicus* cells cultivated under aerobic conditions is depicted in Table 2. Stearic acid (53.6 %) was the dominant fatty acid, followed by palmitoleic acid (22.8 %). The content of other fatty acids, such as palmitic acid, linoleic acid, and nonadecanoic acid, were 13.5%, 6.2 %, and 4.3 %, respectively. Sattari et al. [48] reported linoleic acid as the dominant fatty acid in *M. indicus* cells, while dilute acid hydrolysate of rice straw was used as a carbon source. The length of fatty acids produced in M. indicus cells during fatty acid synthesis can be affected by various factors, such as the feedstock used, the pretreatment applied, the presence of inhibitors in the hydrolysate, and the presence of oxygen [48, 70, 71]. Stearic acid and palmitoleic acid, which are the main fatty acids found in the lipid extracted from *M. indicus* biomass, are particularly suitable for biodiesel production due to their low-temperature fluidity which leads to favorable cold flow and oxidative stability properties [72]. Therefore, the lipid

462 extracted from *M. indicus* cells was transesterified by HCl/methanol to produce biodiesel. The
463 yield of biodiesel was 54 %, according to Eq. 6.

464

465 **Table 2.** The content of fatty acids containing in lipid extracted from *M. indicus* biomass

Fatty acids	Content (% wt)
Palmitic acid (C16:0)	13.5
Palmitoleic acid (C16:1)	22.8
Stearic acid (C18:0)	53.6
Linoleic acid (C18:2)	6.2
Nonadecanoic acid (C18:3)	4.3

466

467 **3.5. Extraction of chitosan and determination of cell wall properties**

468 In addition to intracellular lipids, *M. indicus* cells synthesize chitosan in the cell walls, which is a
469 highly valuable byproduct. Table 3 summarizes the results of chitosan, phosphate, AIM, GLcN,
470 GLcNAc yields, and degree of deacetylation (DD) available in *M. indicus* cells cultivated
471 aerobically in synthetic media and dilute acid hydrolysate of corn stover. According to the results
472 (Table 3), 36 mg g^{-1} chitosan was extracted from *M. indicus* cells cultivated on dilute acid
473 hydrolysate, and 35 mg g^{-1} chitosan was obtained from that cultivated on synthetic media. This
474 result is in the range of extracted chitosan from *M. indicus* cells cultivated on different
475 lignocellulosic feedstocks reported by other researchers [48, 55]. Also, the results of FTIR analysis
476 showed that the extracted chitosan has identical spectra compared to standard chitosan. FTIR
477 spectra are depicted in the Supplementary data.
478 The yield of AIM was 162 mg and 179 mg per gram of *M. indicus* biomass cultivated in dilute
479 acid hydrolysate and synthetic media, respectively. As can be seen in Table 4, the yields of GLcN

and GLcNAc were 0.497 g and 0.175 g per gram of AIM for *M. indicus* cells cultivated in the dilute acid hydrolysate. On the other hand, GLcN and GLcNAc yields were 0.458 g and 0.142 g per gram of AIM for fungal cells cultivated in synthetic media. Generally, the higher amount of GLcN and GLcNAc in the cell wall corresponds to the higher content of chitosan and chitin, respectively [54]. In this work, the higher yield of GLcN than GLcNAc reported in Table 3 showed that chitin has been converted into chitosan during the aerobic cultivation of *M. indicus* cells.

Also, the degree of deacetylation (DD) was 69 % for chitosan extracted from fungal cells cultivated in synthetic media and 65 % for those cultivated in dilute acid hydrolysate, according to Eq. 5. Chitosan DD, an important parameter that determines many biological and physiochemical properties of chitosan, was reported between 60 and 90 % in the related literature [54, 55]. The DD of extracted chitosan in this work is in the range of the reported values for commercial chitosan.

Table 3. The yield of AIM (g g^{-1} biomass), chitosan, GLcNAc, and GLcN (g g^{-1} AIM) for *M. indicus* cells cultivated on synthetic media and dilute acid hydrolysate of corn stover

Major cell wall ingredients yields and main properties	*M. indicus* cultivated in synthetic media	*M. indicus* cultivated in dilute acid hydrolysate of corn stover
AIM yield (g g^{-1} biomass)	0.179±0.002	0.162±0.005
Chitosan yield (g g^{-1} AIM)	0.035±0.002	0.036±0.002
GLcNAc yield (g g^{-1} AIM)	0.142±0.011	0.175±0.021
GLcN yield (g g^{-1} AIM)	0.458±0.043	0.497±0.054
Phosphate (g g^{-1} AIM)	0.118±0.011	0.166±0.002
Degree of deacetylation (DD %)	68.995±0.255	64.788±0.388

3.6. Biogas production

The biomass of *M. indicus* after lipid extraction was subjected to anaerobic digestion in order to produce biogas. To investigate the effect of lipid content on the biogas yield, *M. indicus* cells were also anaerobically digested before lipid extraction. The concentrations (vol basis) of methane and carbon dioxide produced during 40 days are reported in Fig. 5. As seen in Fig. 5, the concentrations of methane and carbon dioxide increased dramatically within 25 days and remained almost constant for the last 15 days.

The results in Fig. 5 showed that 439.4 and 393.9 mL g^{-1} VS methane were produced from whole and de-fatted cells of *M. indicus*, respectively. Also, 214.5 mL g^{-1} VS carbon dioxide was obtained from the anaerobic digestion of fungal biomass and 165.8 mL g^{-1} VS from the anaerobic digestion of defatted ones.

The theoretical methane production yield from pure lipids, proteins, and carbohydrates are 1014, 496, and 415 mL per g of volatile solids, respectively [73]. According to the composition of whole cells of *M. indicus* (lipid 20 %, protein 56 %, and carbohydrates 11 %), the theoretical yields of methane production were 526.2 mL g^{-1} VS and 414.5 mL g^{-1} VS for whole and defatted cells (protein 66 % and carbohydrate 21 %) respectively. According to Fig. 5-b, 83 % of the theoretical methane production yield was achieved from de-fatted cells in this study. Karimi et al. [74] reported a methane production yield of 157.4 mL g^{-1} VS for fungal biomass while blended with lignocellulosic feedstock. The comparatively higher biomethane yield in the present study may be related to the favorable effects of the carbon/nitrogen nutrient requirement available in the fungal cell wall, i.e., GLcNAc, GLcN, and proteins.

So far, there have not been reported results that presented data for biogas production from de-fatted *M. indicus* cells.

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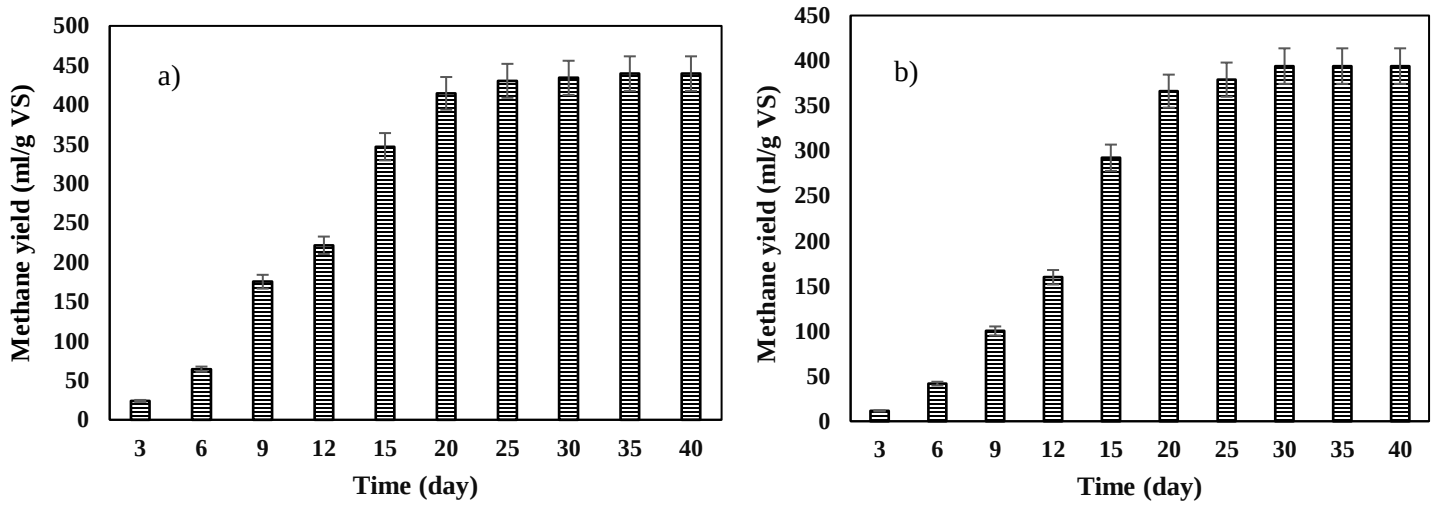

520

521 **Fig. 5.** The yield of methane (ml/g VS) from whole cells of *M. indicus* (a) and defatted ones (b).

522

523 **3.7. Characterizing pretreated and enzymatically hydrolyzed corn stover as animal feed**

524 Standard parameters used to characterize animal feed that were measured for corn stover before
525 and after pretreatment and hydrolysis are summarized in Table 4. According to the results, corn
526 stover lost a considerable amount of carbohydrates after pretreatment and enzymatic hydrolysis.
527 For example, the ADF content, which consists of cellulose and lignin, was 44.8 % for the pretreated
528 and hydrolyzed corn stover compared to only 18.4 % for the raw corn stover. In addition, the lignin
529 content increased 5.5 times compared to the raw corn stover.

530 On the other hand, the lipid and protein content, which are essential parameters in the assessment
531 of animal feed, significantly increased. The lipid was nine times higher and the protein content
532 was twice as high in the pretreated and hydrolyzed corn stover compared to the raw corn stover.
533 However, the energy content of the pretreated and hydrolyzed corn stover, as determined by
534 parameters such as digestible energy (DE), metabolizable energy (ME), and net energy lactation

535 (NEL_{3x}) was 2.3, 1.8, and 1.1 Mcal kg^{-1}, respectively, while these parameters for raw corn stover
536 were 2.9, 2.5 and 1.5, respectively. Nevertheless, due to the high content of lignin in pretreated
537 and hydrolyzed corn stover, it is suggested to use it as a feed that has low nutritional value, such
538 as straw.

539 **Table 4.** Standard parameters of animal feed for pretreated and hydrolyzed corn stover vs. raw
540 corn stover

Parameter	Content		Unit
	Hydrolyzed corn stover	Untreated corn stover	
Dry matter	96.5	93.5	% wt
Crude protein	15.4	7.7	% wt
Ether Extract	9.3	1.9	% wt
Acid detergent insoluble crude protein (ADICP)	5.7	0.6	% wt
Natural detergent insoluble crude protein (NDICP)	6.6	2.6	% wt
Acid detergent fiber (ADF)	44.8	18.4	% wt
Natural detergent fiber (NDF)	49.5	39.5	% wt
Acid detergent lignin (ADL)	27.6	5.0	% wt
Non-fibrous carbohydrates (NFC)	24.0	47.5	% wt
Total digestible nutrient (TDN)	51.2	66.5	% wt
Ash	8.4	6.1	% wt
Digestible energy (DE)	2.3	2.9	Mcal kg^{-1}
Metabolizable energy (ME)	1.8	2.5	Mcal kg^{-1}
Net energy lactation (NEL_{3x})	1.1	1.5	Mcal kg^{-1}

541

542

3.8. Mass balance and biorefinery products classification

In accordance with the biorefinery concept, corn stover was used to produce multiple high-value products, and the mass balance results are shown in Figure 6. One kg of corn stover, containing 493 g glucan, 112 g xylan, 123 g lignin, and 57 g ash, was subjected to dilute acid pretreatment. After dilute acid pretreatment, 99 g xylose and 293 g glucose were released in the liquid phase, while 280 g glucan and 3 g xylan remained in the solid phase. The solid phase was hydrolyzed enzymatically to achieve 197 g glucose and then followed by the production of 74 g ethanol and 2 g glycerol in the anaerobic cultivation of *M. indicus* cells. After anaerobic cultivation, the biomass of *M. indicus* was separated and subjected to dilute acid hydrolysate in the aerobic condition, which resulted in 129 g ethanol, 3.5 g glycerol, and 300 g fungal biomass. The obtained fungal biomass was divided into two parts with the same weights. One part was digested in an alkaline solution, resulting in 870 mg chitosan, while another was de-fatted and converted to 16 g biodiesel in HCl/MeOH. Finally, defatted fungal biomass was subjected to anaerobic digestion and produced 2.8 L methane.

Other studies that investigated the use of microbial lipids to create biodiesel [75, 76] have suggested using multiple types of microorganisms to produce biofuels. However, the current biorefinery has the advantage of using just one type of microorganism to produce all biofuels, as well as several valuable byproducts such as chitosan, glycerol, and animal feed. The viability of commercial biofuel production from corn stover is largely dependent on the production of these additional byproducts [3], making this integrated biorefinery a potentially suitable option."

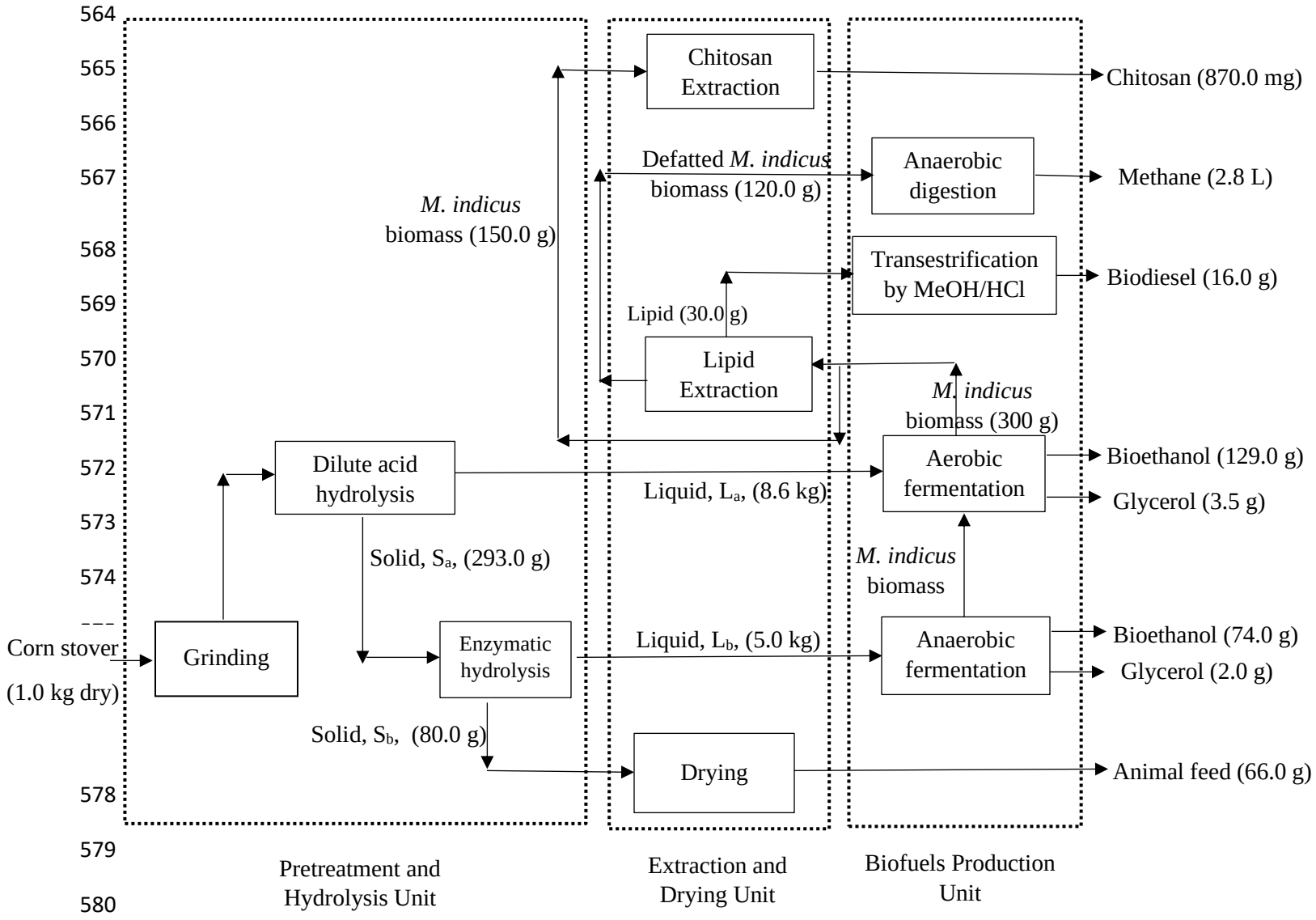

Fig. 6. Mass balance over biorefining of corn stover by using *M. indicus* (based on the ~~best~~ results obtained)

3.9. Socioeconomic analysis

In Iran, a total amount of 2.7 million tons of corn stover was cultivated on 261,119 hectares in 2020 [77]. In most cases, corn stover, as animal feed, is mainly harvested at 80-95% efficiency [78]. Therefore, it could be estimated the residue-to-product ratio (RPR) of ~0.10, leading to

589 remain 1.27 million tons of corn stover on the farmland as residue. According to Eq. (9-10) and
590 the factor of ground cover (f_{GC}, 2.7 t ha^{-1}) and residue collection efficiency (f_{CR}, 0.35) [13], 197,742
591 tons corn stover residue are collectable for biorefinery. By considering moisture content (f_m, 0.2 w
592 w^{-1}), the amount of 158,194 tons dry corn stover residue is available for biorefining. The estimated
593 biofuels, saved corresponded fossil fuels, and greenhouse gas reduction were subsequently
594 determined and summarized in Table 5. Given the biofuel yields described in Figure 6, the annual
595 volumes of bioethanol, biodiesel, and biogas production calculated using Eq. (11) are 40.7 million
596 litters, 2.8 million m^3, and 449.2 million m^3, respectively. In the case of biodiesel, CO_2 emission
597 can be diminished by 2.7 million tons, whereas it could be reduced by 1.5 million tons for biogas
598 production. Also, the ethanol produced in this biorefinery could prevent 58.3 thousand tons of CO_2
599 emission into the atmosphere. The data (Fig. 7) also shows that substituting biodiesel and
600 biomethane for corresponding fossil fuels significantly reduced SCC by 127.7 million dollars and
601 67.3 million dollars, respectively, while the reduction in SCC for replacing gasoline with
602 bioethanol was estimated to be 2.6 million dollars.

603 **Table 5.** The potential of produced biofuels from collectible corn stover residues and the amount
604 of saved corresponded fossil fuels as well as a reduction in GHG emissions in Iran[a]

Biofuels	Produced biofuels	Saved corresponded fossil fuel [b]	GHG reduction (million tons)
Bioethano (million liters)	40.7	27.7	0.0583
Biodiesel (million m^3)	2.8	2.6	2.7
Biomethane (million m^3)	449.2	449.2	1.4

605 [a] Well to wheel CO_2 emission for biomethane and ethanol estimated to be 2.44 kg CO_2/m^3 methane [79], 1.91 kg CO_2/l
606 biodiesel [60] and 0.6 kg CO_2/l ethanol [60]. Well to wheel CO_2 emission for corn stover was estimated to be zero due
607 to considering as zero waste.
608
609 [b] A value of *R* equal to 0.68 l gasoline l^{-1} cellulosic ethanol, 0.91 l diesel l^{-1} biodiesel, and 1.00 m^3 biomethane m^3
610 methane [60]
611

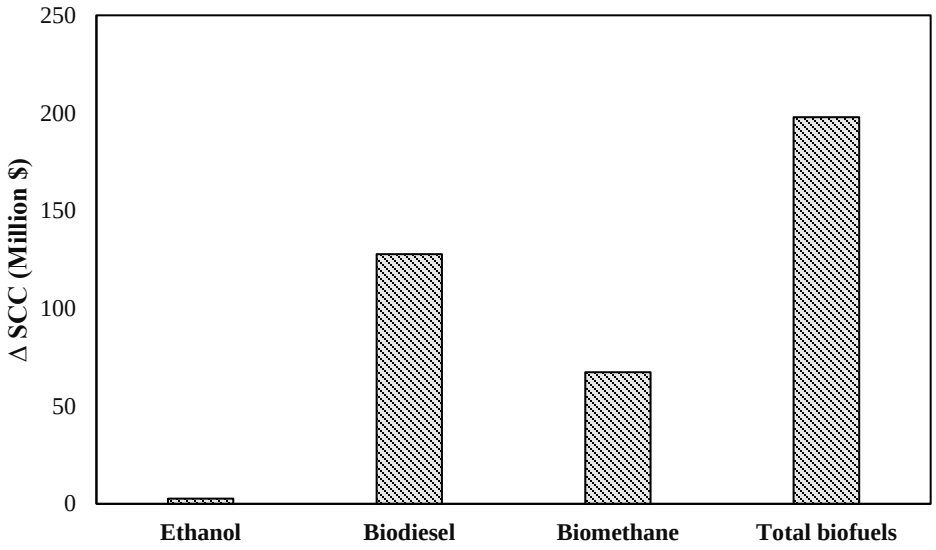

Fig. 7. Substituting bioethanol, biodiesel, and biomethane for fossil fuels in Iran's transportation sector for the reduction in the total social cost of carbon dioxide (Δ_{SCC}).

5. Conclusions

This study presents an integrated biorefinery for producing biofuels and valuable byproducts from corn stover via the integration of dilute acid pretreatment, enzymatic hydrolysis, and fermentation with a single microorganism. One of the main advantages of this biorefinery is its low acid consumption and short resident reaction time, as well as its ability to use non-detoxified hydrolysate because the *M. indicus* cells used in this process are resistant to inhibitor compounds present in the acid hydrolysate. The biorefinery approach outlined in this study also demonstrated a significant reduction in GHG emissions and a reduction in social costs. However, to have a complete circular process, complementary optimizations regarding the minimization of waste streams produced during the pretreated biomass naturalization, biodiesel and biogas production, as well as lipid and chitosan extraction are necessary. Besides, the corrosive properties of acids on material constructions along with the cost of enzymes are other challenges that may hinder the development of this biorefinery. Recycling and reusing (bio)chemicals used in the process, e.g., solvents, enzymes, and washing water, should be employed to reduce the negative impacts of produced wastes. Also,

performing the dilute acid pretreatment at a high solid loading can be reduced the corrosive effects of acids on the constructions. Moreover, further studies, including techno-economic analysis and life cycle assessment (LCA), are needed to comprehensively evaluate the potential of this biorefinery for commercialization.

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