Comparative Study on the Usability of Lignocellulosic and Algal Biomass for Production of Alcoholic Fuels

Faryal Kabir,[a,*] Muhammad Gulfraz,[a] Ghazala Kaukab Raja,[a] Muhammad Inam-ul-Haq,[b] Iram Batool,[a] Muhammad Awais,[c] Umme Habiba,[a] and Hina Gul [a]

The aim of this study was to develop an approach for the processing of agricultural and organic wastes to produce alcoholic fuels such as ethanol and butanol. The cellulosic materials wheat straw (WS), rice straw (RS), and corn stover (CS) were pretreated with dilute sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH) individually, and microalgae (*Chlorella vulgaris*) was treated with dilute H_2SO_4 and then fermented. The results indicated that pretreatment in acidic condition was best to produce fermentable sugar. However, the high glucose concentration was achieved in *C. vulgaris* (32 g/L) and WS (20.6 g/L) among lignocellulosic biomass. The microalga that was grown in the nutrient deficient condition had a carbohydrate content of 51% ± 2.1. After fermentation, high concentration of ethanol 9.5 g/L (yield 93.7%) and butanol contents of 7.4 g/L (yield 91.3%) were recorded in wheat straw, whereas *C. vulgaris* yielded ethanol and butanol concentrations of 14 g/L and 11.8 g/L respectively. The results may help to increase the production of biofuels and reduce the need for imported fuels.

Keywords: Biomass; Treatment; Fermentation; Bioproducts

*Contact information: a: Department of Biochemistry, University Institute of Biochemistry & Biotechnology, PMAS Arid Agriculture University, Rawalpindi, Punjab 46000, Pakistan; b: Department of Plant Pathology, PMAS Arid Agriculture University, Rawalpindi, Punjab 46000, Pakistan; c: Department of Biosciences, COMSATS University Islamabad, Sahiwal Campus, Off GT road, 57000, Sahiwal, Pakistan; * Corresponding author: faryal_kabeer13@yahoo.com*

INTRODUCTION

Due to increasing concerns regarding energy security and climate change, there has been an increased exploration of various sources for alternate energy. The transportation sector is a significant contributor to the emission of greenhouse gases because of the utilization of fossil fuels. However, the replacement of oil-derived fuels with biofuels, such as ethanol, can reduce the environmental impacts and provide social and economic benefits (Humbird *et al.* 2011). Various alternative routes to generate sustainable biofuels from biomass have been investigated. Some important biological energy resources are bioelectricity, biogas, biodiesel, and bioalcohol. Among these sources, bioalcohol has great potential to reduce the emission of greenhouse gases, decrease the dependence on fossil fuels, and act as a potential fuel for the transportation sector (Dhamole *et al.* 2015). The worldwide production of bioethanol has increased dramatically because many countries are trying to reduce the import of oil to improve the air quality and grow rural economics. The global ethanol production was 51,000 million liters by the end of 2007 (Renewable Fuel Associations 2007). The fuel covered 73% of produced ethanol, whereas industrial and beverage ethanol comprise of 10% and 17%, respectively (Sanchez and Cardona 2008). Ethyl alcohol has some advantages as a fuel because it has higher oxygen content.

The high oxygen level of ethyl alcohol allows for the improved oxidation of hydrocarbons with the successive reduction in aromatic compounds and carbon monoxide emissions. Moreover, ethanol has better octane rating properties (Thomas and Kwong 2001).

Biomass is a vital energy source in Pakistan because it is an agricultural based country. The biomass produced in the livestock and agriculture sector is in the form of animal waste, sugarcane bagasse, and rice husk crop residue (Chaudhry *et al.* 2009; Amiri *et al.* 2014). Second generation biomass is mainly composed of lignocellulosic material. Lignocellulosic biomass is the most plentiful organic substance that contains cellulose (35% to 50%), hemicellulose (20% to 35%), and lignin (5% to 30%) (Huber *et al.* 2006). Various renewable energy resources are agricultural substances including green leaves, fruit shells, straws, nut shells, and fruit seeds (Demirbaş 2001). The most commonly used feedstocks are wheat straw, wheat bran, corn stover, corn steep liquor, and apple pomace (Ejezi *et al*. 2007). Currently, agricultural waste is more often used to produce biofuels, such as bioethanol, biodiesel, biohydrogen, and methane, than energy crops because it is an inexpensive and highly accessible substrate that can minimize the cost of production and has no effect on food prices (Valentine *et al.* 2012). However, the energy crops still have a competition with food crops for use of agricultural land, and ethically these are considered as non-sustainable resources (Thompson and Meyer 2013). Because a large amount of agrowaste is generated and discarded, an alternative option is to utilize such lignocellulosic biomass to reduce the competition between fuel and food (Mahro and Timm 2007). Therefore, the second generation feedstocks are entirely non-food in nature and are regarded as superior over first generation feedstocks (Thompson 2012; Valentine *et al.* 2012).

The price of the substrate used for fermentation is a major factor that affects the economics of the production of biofuels such as butanol (Qureshi and Blaschek 2000). Wheat straw is a widely available material all over the world, but it is often burned in the fields as its disposal. The carbon monoxide released from this process increases air pollution and negatively affects human health (Dale *et al.* 1996; Zhu *et al.* 2006). As a domestic residue, rice straw has a great potential to produce biofuels such as acetone, butanol, and ethanol (Nimcevic and Gapes 2000). Corn stover is a lignocellulosic biomass that is an ideal material to produce fuels and chemicals, due to its low cost, environmental advantages, and economic advantages. Approximately 75% to 80% of corn stover is composed of hemicellulose and cellulose, which are not easily transformed into simple sugars due to their recalcitrant nature (Esteghalian *et al.* 1997; Kalman *et al.* 2002; Kadam and McMillam 2003). Third generation biofuels are usually produced from microalgae (Nigam and Singh 2011). However, the main challenge is to minimize the consumption of energy for ethanol production from microalgae at an industrial level as evaluated by Medeiros *et al.* (2015). In this context, biorefinery has been introduced as a great concept to produce various products from algal biomass, but the information regarding this area is limited (Cherubini 2010; Cuellar-Bermudez *et al.* 2015). Most of the studies focused on biodiesel production from microalgae because of high lipid contents (Alvira *et al.* 2010). Moreover, some strains of microalgae such as *Chlorella, Scenedesmus, Chlamydomonas*, and *Dunaliella* have carbohydrate contents as high as 50% dry cell weight under specific cultivation conditions and are known as promising candidates for ethanol production (Ho *et al.* 2012; Chen *et al.* 2013). The utilization of carbohydrate-rich biomass for ethanol production is beneficial because some species have a higher carbon dioxide fixation and growth rate than terrestrial plants. The microalgae has low lignin contents, therefore saccharification is quite easy as compared to lignocellulosic biomass as it requires mild

pretreatment conditions (Harun *et al.* 2010; Chen *et al.* 2013; Passos *et al.* 2014). The pretreatment process by using dilute acid could yield high sugar contents in microalgae (Ho *et al.* 2013).

In this study, agricultural waste samples and native microalgae isolate were used as substrates for butanol and ethanol production by fermentation with *Clostridium acetobutylicum* and *Saccharomyces cerevisiae*, respectively. This research aimed to 1.) Optimize the treatment conditions needed to break down the biomass into simple sugars by using various enzymes; and 2.) Produce ethanol and butanol from cellulosic biomass through the fermentation process.

EXPERIMENTAL

Materials

Collection of the substrates

The wheat straw and corn stover samples were collected from the Bagh district of Azad Kashmir, Pakistan, and the rice straw was collected from the Gujrat district of Punjab, Pakistan. The microalgae (*Chlorella vulgaris*) was isolated from wastewater in Rawalpindi, Pakistan and cultivated in BG-11 medium in the presence of light at 27 °C. The BG-11 medium was prepared according to Feng *et al.* (2011b). The agrowaste samples were dried in sunlight for 2 to 3 days and then placed in an oven at 60 °C for 24 h. The samples were ground using a lab grinder (mortar grinder RM 200; Shanghai SAM Company, Shanghai, China), filtered through an 80 mm-mesh sieve, packed in sealed plastic zip lock bags (Flexible Packaging Company, Lahore, Pakistan), and stored at 4 °C until further analysis.

Methods

Proximate analysis

The proximate analysis of the biomass was conducted in the laboratory, and the samples (wheat straw, rice straw, corn stover, and *C. vulgaris*) were analyzed for their wet and dry weight, crude fiber content, crude protein content, crude fat content, and ash content (AOAC 1990).

Determination of the cellulose, hemicellulose, and lignin contents

The cellulose and hemicellulose content of the samples were estimated according to the standard method reported by Haifeng *et al.* (2015). The lignin content was calculated according to Kovacs *et al*. (2009).

Reducing sugar concentration by spectrophotometer

The reducing sugar concentration was determined *via* the 3,5-dinitrosalicylic acid reagent (DNS) method (Sigma-Aldrich, St. Louis, MO, USA) (Miller 1959).

Fatty acid analysis using gas chromatography (GC)

The fatty acids in the microalgal lipid were analyzed using a GC-flame ionization detector (FID; Clarus 500 Gas Chromatograph; Perkin Elmer; San Francisco, CA, USA) equipped with a Nukol column (Sigma-Aldrich, St. Louis, MO, USA). Helium was used as the carrier gas and the initial temperature of the oven was set to 110 °C. The oven temperature was increased to 220 °C at a rate of 10 °C/min. The temperature of the injector

and the detector were adjusted at 250 °C and 220 °C, respectively. The procedure for the analysis was followed according to Lam and Lee (2013).

Analysis of the proteins, carbohydrates, and lipids

The carbohydrates and proteins contents in biomass were determined by phenol sulphuric acid method and Bradford assay respectively (Guldhe *et al*. 2016; Ramsundar *et al*. 2017). The total lipids were extracted from algal biomass by one step solvent extraction procedure proposed by Sires and Brillas (2012).

Experimental Design

Pretreatment

For the acidic and basic pretreatment, the wheat straw, rice straw, and corn stover samples were first washed with tap water and then dried in sunlight followed by a vacuum drying oven (Sugold, Ningbo, China) for further treatment. The dilute sulfuric acid (H_2SO_4) was added to the biomass at a 20% solid to liquid ratio (w/v) dose based on solids at concentrations of 0.5%, 1%, and 1.5% (v/v) for the acidic pretreatment (Saha *et al*. 2005). For dilute alkali pretreatment the sample at a solid loading of 20% (w/v) was treated with dilute sodium hydroxide (NaOH) at concentrations of 0.5%, 1%, and 1.5% (v/v). The treated samples were autoclaved (Hirayama HVE-50 upright autoclave, Hirayama, Japan) at three different temperatures (100 °C, 110 °C, and 121 °C) with reaction times of 10 min, 15 min, and 20 min. The pH of the pretreated sample was adjusted to 5.0 with 1 M HCl and 1 M NaOH before the enzymatic saccharification process (McIntosh and Vancov 2011).

Enzymatic hydrolysis

After the pretreatment process, the acidic and basic pretreated material was allowed to cool down to be used for further hydrolysis. Sodium citrate buffer (50 mM) at a pH of 5.0 was prepared and added in a reaction mixture to maintain the pH. An ampicillin (100 µg/mL) and Augmentin (100 µg/mL) antibiotic solution was also added to inhibit the growth of microorganisms. Cellulase and β-glucosidase enzymes were added in the pretreatment mixture at a loading of 0.1 mL/g substrate to hydrolyze the cellulose and hemicellulose into glucose. The pretreated samples were placed in an orbital shaker incubator (ES-20/60; Biosan Laboratories Inc., Warren, MI, USA) at 50 °C and 200 rpm for 72 h. The cellulase (Celluclast 1.5 L; Novozymes, Bagsværd, Denmark) and Novozyme 188 (Novozymes, Bagsværd, Denmark) had activities of 700 EGU/g (60 FPU/g) and 250 CBU/g, respectively. The enzymes were loaded in a reaction mixture at a ratio of 3:1 for Celluclast and Novozyme 188, respectively (Lu *et al*. 2012).

Evaluation of the growth rate of microalgae and biomass harvesting

The *C. vulgaris* was grown in a BG-11 medium (control) and nutrient deficient medium, where sodium nitrate ($NaNO_3$) was removed from the BG-11 medium to enhance the carbohydrate concentration in the algal biomass. The growth of the microalgae was measured by following the method of Zhou *et al*. (2012), and the biomass productivity was determined using Eq. 1 (Feng *et al.* 2011a),

$$P_{\text{Biomass}} = (DW_{\text{x}} - DW_1) \quad (1)$$

where P_{Biomass} represents the biomass productivity (g/L/day), DW_{x} represents the biomass concentration based on the dry weight (g/L) at the initial time of cultivation, and DW_1 is

the concentration based on the dry weight (g/L) at the end of the cultivation period. After a 7 d cultivation period, the biomass was harvested *via* centrifugation (LCEN-200/201; MRC Medical Consulting Ltd., Beijing, China) at 3,000 rpm for 10 min (Barsanti and Gualtieri 2006).

Acidic Treatment of the C. vulgaris

After the cultivation of *C. vulgaris* in the nutrient-starved medium, the harvested algae biomass was treated with sulfuric acid. The freeze-dried microalgae powder was mixed with dilute sulfuric acid (H_2SO_4) at a final acid concentration of 1% (v/v) and heated at 121 °C for 20 min. After the hydrolysis process, the algal suspension was cooled, neutralized with NaOH to a pH of 6.0, and centrifuged at 4,000 rpm for 10 min. The supernatant was used to analyze the sugar contents and for the fermentation process.

Fermentation

The fermentation process was performed as a separate hydrolysis and fermentation (SHF). The best pretreatment conditions (hydrolysates obtained from the dilute acid pretreatment and by enzymatic hydrolysis) of the high glucose yields were further fermented. The fermentation experiments were performed *via* the addition of 5 g/L yeast *Saccharomyces cerevisiae BY4741* (*MATa)* (EUROSCARF, Frankfurt, Germany) or 5 g/L bacteria culture *Clostridium acetobutylicum* ATCC 824 American Type Culture Collection (ATCC; Cedarlane Labs, Ontario, Canada) into the pretreated hydrolysates. The process was conducted at 32 °C and 120 rpm for 72 h under anaerobic conditions.

Yeast strain and preparation of the inoculum

Baker yeast (*S. cerevisiae*) was selected for the ethanol production, and it was preserved in vials containing glycerol at -20 °C. The yeast was inoculated in a 250-mL Erlenmeyer flask that contained 100 mL of yeast peptone dextrose (YDP) growth medium at a pH of 4.5. This medium was composed of yeast extract (10 g/L), glucose (70 g/L), and peptone (50 g/L) (Yu *et al.* 2007). The flask was sealed with a cotton plug and was autoclaved at 121 °C for 15 min. The cultures were incubated on a rotary shaker (ES-20/60; Biosan, Warren, MI, USA) for 48 h at 32 °C and 120 rpm. To increase the cell concentration (2%), the yeast was transferred into a 500-mL Erlenmeyer flask with 200 mL of YDP medium. The flasks were incubated under the previously mentioned conditions. After 48 h, the cells were harvested and used as inoculum for the fermentation experiment.

Bacterial strain and preparation of the inoculum

Spores of *C. acetobutylicum* were maintained in distilled water at 4 °C and used for butanol production. To prepare the inoculum, reinforced clostridial medium (RCM) was used. The RCM medium contained glucose (5.0 g/L), starch (1.0 g/L), peptone (10.0 g/L), yeast extract (3.0 g/L), beef extract (10.0 g/L), sodium chloride (5.0 g/L), sodium acetate RCM (3.0 g/L), and cysteine hydrochloride (0.5 g/L). The pH was adjusted to 6.5 ± 0.1. The mixture was autoclaved at 121 °C for 15 min and 100 mL of the medium was inoculated in a 250-mL screw capped Erlenmeyer flask. The culture was incubated on the rotary shaker at 120 rpm for 72 h at 37 °C to be inoculated into an alcohol production medium (Ranjan and Moholkar 2011).

Fractional distillation

After the fermentation process, the mixture contained several alcohols such as methanol, ethanol, butanol, and acetone. These alcohols were removed *via* fractional distillation in a fractional distillation apparatus (Quickfit SH4/33; SciLabware Stoke-on-Trent, Midlands, UK). The distillation process separated the butanol by its higher boiling point. Because butanol has a higher boiling point (118 °C) than water (100 °C), the water vaporized before the collection of butanol. The butanol was then condensed and separated. The boiling point of ethanol (78.3 °C) is lower than that of water, so it was able to condense earlier than water. The concentration of the ethanol and butanol was measured by procedure proposed by Maiti *et al.* (2015).

Statistical analysis

The data were statistically analyzed using an analysis of variance (ANOVA) by MSTAT-C software, version 6.0 (Michigan State University, East Lansing, MI, USA) to select the optimum pretreatment conditions. GraphPad prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for analysis of the standard deviation and growth curve of microalgae.

RESULTS AND DISCUSSION

Chemical Compositions of the Biomass Samples

Various biomass samples wheat straw (WS), rice straw (RS), corn stover (CS), and *Chlorella vulgaris* (CV) were analyzed for quantification of lignocellulosic components and other extractives on the basis of percent dry weight, as shown in Table 1. The total dry matter in WS, RS, CS, and CV was 92.8, 90.8, 91, and 91.6 wt%, respectively. It was reported earlier that in lignocellulosic biomass, the total of cellulose, hemicellulose, and lignin comprises about 90 wt% dry matter, while the remaining material consists of other extractives and ash contents (Lehtomaki *et al.* 2007). The cellulosic contents were higher (39.6 wt%) in WS and lower in CS (32.2 wt%) among lignocellulosic biomasses, whereas, *C. vulgaris* has the highest cellulosic material (42.8 wt%) as well as crude fat contents (28.4 wt%) and lower protein contents than all of the feedstock samples. This may be because of limited concentration of nitrogen in culture medium of algae. Other studies also have shown that growing microalgae in a nitrogen deficient medium can produce a high amount of carbohydrates and lipids because accumulated proteins in microalgae cell can be converted into carbohydrates or lipids (Dragone *et al.* 2011; Rismani-Yazdi *et al.* 2011; Yeh and Chang 2011; Ho *et al.* 2012). The hemicellulose concentration ranged from 28.6 to 8.4 wt% with CS representing highest level. The high hemicellulose content was recorded in fast growing plants due to which mineralized solutions containing chlorides, silicic acid, sulphates, and nitrates can be transported in plants (Li *et al.* 2014). CS also contain higher level of lignin (18.1 wt%), and this is in accordance with earlier study (Saini *et al.* 2015).

Pretreatment of the Agricultural Substrates and the Sugar Analysis after Enzymatic Hydrolysis

The three agrowaste samples underwent the pretreatment process at optimized conditions followed by enzymatic hydrolysis in a 500-mL Erlenmeyer flask at 50 °C for up to 3 days to produce sugar. Pretreating lignocellulose breaks down the lignin and increases the accessibility of enzymes and microbes for the carbohydrates.

Table 1. Proximate Analysis of the Samples on the basis of Dry Biomass

Parameters	Wheat Straw	Rice Straw	Corn Stover	*C. vulgaris*
Dry Matter (%)	92.8 ± 0.3	90.8 ± 0.0	91 ± 0.5	91.6 ± 0.6
Moisture Content (%)	7.2 ± 0.2	5.4 ± 0.1	5.3 ± 0.2	6.1 ± 0.1
Volatile Matter (%)	88.3 ± 0.1	88.7 ± 0.1	85.2 ± 0.1	79.5 ± 0.4
Fixed Carbon Content (%)	18.4 ± 0.9	16.2 ± 0.1	14.1 ± 0.3	14.0 ± 0.01
Ash Content (%)	4.5 ± 0.0	2.1 ± 0.0	5.8 ± 0.1	12.1 ± 0.8
Crude Fiber Content (%)	15 ± 0.4	11 ± 0.4	2.5 ± 0.1	3.2 ± 0.4
Crude Fat Content (%)	3.6 ± 0.1	1.9 ± 0.1	2.9 ± 0.5	28.4 ± 0.0
Crude Protein Content (%)	11.5 ± 0.6	4.3 ± 0.1	7.1 ± 0.1	12.5± 0.1
Cellulose Content (%)	39.6 ± 0.9	33.0 ± 0.1	32.2 ± 0.0	42.8 ± 0.3
Hemicellulose Content (%)	27.9 ± 0.2	28.6 ± 0.0	25.7 ± 0.1	8.4 ± 0.1
Lignin Content (%)	13.1 ± 0.1	14.1 ± 0.2	18.0 ± 0.3	ND

Data represented in the above table as ± SD (standard deviation, n=3) and on the basis of dry weight (%)

Two pretreatment methods were applied on the substrates. For the physical pretreatment process, these substrates were first ground to a fine powder and passed through 80-mm mesh sieve to reduce the size of the particles. The substrates were then subjected to chemical pretreatment. The samples were treated by using different concentrations of dilute H_2SO_4 (0.5%, 1%, and 1.5%) and dilute NaOH (0.5%, 1%, and 1.5%) and then heated at 100 °C, 110 °C, and 120 °C, at retention times (T_r) of 10 min, 15 min, and 20 min. The reactions were performed in triplicate. For each temperature, the substrate was pretreated with three different concentrations of H_2SO_4 and NaOH. Three treatments of one sample were pretreated at one temperature and one reaction time. A total of nine experiments were performed, so 81 treatments (9 × 9 = 81) of three samples were performed at three different temperatures to check the optimum condition for the acidic pretreatment and also for alkaline pretreatment separately. The highest values obtained are in bold.

The glucose yields from WS, RS, and CS after treating with different H_2SO_4 concentrations and enzymatic hydrolysis are illustrated in Table 2, and the treatments of NaOH followed by enzymatic hydrolysis are shown in Table 3. During the pretreatment process, the dilute H_2SO_4 had a higher sugar yield than NaOH treated samples. Among all raw materials used, a maximum amount of sugar (20.6 g/L± 0.1 g/L) was found in the wheat straw when the sample was treated with 1.5% H_2SO_4 at 120 °C for 20 min. It was observed during experiments that the amount of sugar was increased by increasing the concentration of H_2SO_4 from 0.5% to 1.5%. In terms of highest glucose yield, following sequence was observed WS (20.6 g/L) > CS (17.8 g/L at 120 ºC for 15 min) > RS (15.4 g/L at 120 °C for 20 min).

Table 2. Chemical Pretreatment of the Agrowaste Samples with Dilute H_2SO_4 to Release Sugars After 72 h

Acid (%)	Temp (°C)	Time (min)	Glucose Concentration (g/L)		
			Wheat Straw	Rice Straw	Corn Stover
0.5	100	10	11.0 ± 0.3	8.4 ± 0.3	15 ± 0.1
		15	11.0 ± 0.0	10.8 ± 0.0	15.1 ± 0.8
		20	11.4 ± 1.2	14.2 ± 0.4	14.2 ± 0.6
	110	10	4.1 ± 1.0	7.6 ± 0.1	10.9 ± 0.4
		15	5.4 ± 0.7	8.7 ± 0.7	11.8 ± 0.1
		20	5.9 ± 0.1	11.4 ± 0.1	13.4 ± 0.0
	120	10	11.7 ± 0.6	13.2 ± 1.3	15.8 ± 0.8
		15	12.8 ± 0.1	14.8 ± 0.7	14.0 ± 0.0
		20	16.7 ± 0.1	12.9 ± 1.2	15.0 ± 0.7
1	100	10	11.7 ± 0.1	13.7 ± 0.8	14.9 ± 0.1
		15	13.7 ± 0.8	13.9 ± 0.4	16.7 ± 0.3
		20	15.9 ± 1.3	13.9 ± 0.3	16.0 ± 0.5
	110	10	7.7 ± 0.6	17.0 ± 0.7	17.1 ± 0.1
		15	11.4 ± 0.0	14.3 ± 1.2	14.7 ± 0.6
		20	8.7 ± 0.9	12.5 ± 1.8	14.5 ± 0.5
	120	10	13.2 ± 0.1	12.5 ± 0.4	15.6 ± 0.1
		15	15.5 ± 0.1	12.6 ± 0.6	15.4 ± 0.3
		20	17.4 ± 0.2	12.9 ± 0.1	16 ± 1.2
1.5	100	10	13.4 ± 0.5	13.6 ± 0.0	16.7 ± 0.1
		15	14.2 ± 1.4	14.3 ± 0.0	15.9 ± 1.2
		20	14.5 ± 1.2	11.9 ± 0.8	15.6 ± 0.1
	110	10	10.2 ± 0.6	17.7 ± 0.4	14.6 ± 0.3
		15	11.9 ± 0.2	14.0 ± 0.1	15.6 ± 0.8
		20	13.9 ± 0.9	15.2 ± 0.6	15.2 ± 0.9
	120	10	13.9 ± 0.1	14.7 ± 0.1	15.7 ± 0.1
		15	17.5 ± 0.5	14.9 ± 0.3	**17.8 ± 0.1**
		20	**20.6 ± 0.1**	**15.4 ± 0.2**	15.7 ± 0.3

Data represented in the above table as ± SD (standard deviation, n=3)

Compared to acidic (H_2SO_4) pretreatment, the alkaline (NaOH) pretreatment resulted in glucose concentration of 17.7 g/L (1% NaOH, 120 °C and 15 min), 10.9 g/L (0.5% NaOH, 100 °C and 20 min) and 15.4 g/L (1.5% NaOH, 100 °C and 20 min) in WS, RS and CS respectively under optimized conditions.

However, a high glucose yield could be obtained with a higher concentration of H_2SO_4 and a longer reaction time, while temperature is the most important factor to achieve a high amount of sugar. Therefore, a moderate temperature and acid concentration are two key factors that determine the glucose contents of different pretreatment conditions. Similar findings were reported by Grohmann *et al.* (1995) with orange peel hydrolyzed by acid at a low temperature. The hydrolysis rate was highest during the initial 24 h of enzymatic hydrolysis, and it decreased gradually over the next 48 h to 72 h. The decreased rate of hydrolysis was due to the end product inhibition of the sugar produced as a result of the enzymatic action.

It is likely that the released sugar can be converted into fermentation inhibitors and other products (Garcia *et al.* 2011). In one of the related studies of wheat straw it was reported that after acidic pretreatment, the cellulose as well as lignin contents increased from 36.6 to 69.8 % and 22.2 to 26.4 %, respectively, whereas the hemicellulose contents were reduced to 3.8%. Moreover, 12.1 g/L glucose and 45 g/L xylose were recorded. The

fermentation inhibitors such as 5-hydroxymethylfurfural (0.2 g/L), acetic acid (1.4 g/L) and furfural (0.4 g/L) were also observed in high concentration (Agrawal *et al.* 2015a).

Table 3. Chemical Pretreatment of the Agrowaste Samples with Dilute NaOH to Release Sugars After 72 h

Alkali (%)	Temp (ºC)	Time (min)	Glucose Concentration (g/L)		
			Wheat Straw	Rice Straw	Corn Stover
0.5	100	10	12.6 ± 0.6	5.2 ± 0.7	3.9 ± 0.6
		15	12.9 ± 0.1	9.1 ± 0.1	5.6 ± 0.4
		20	14.0 ± 0.1	**10.9 ± 1.2**	10.1 ± 0.1
	110	10	14.2 ± 0.3	4.4 ± 1.0	12.9 ± 0.3
		15	14.5 ± 0.9	4.7 ± 0.1	13.1 ± 0.4
		20	15.6 ± 0.1	5.0 ± 0.5	9.4 ± 0.7
	120	10	13.5 ± 0.1	4.6 ± 0.6	13.7 ± 0.1
		15	16.6 ± 0.3	5.2 ± 0.0	14.1 ± 0.1
		20	12.5 ± 0.6	5.6 ± 0.2	14.8 ± 0.3
1	100	10	11.1 ± 0.1	4.6 ± 0.9	13.6 ± 0.1
		15	9.4± 0.0	5.4 ± 0.1	13.9 ± 0.4
		20	11.0 ± 0.3	9.9 ± 0.1	15.2 ± 0.3
	110	10	14.8 ± 0.5	3.9 ± 0.01	13.2 ± 0.0
		15	14.7 ± 0.5	4.1 ± 0.9	9.2 ± 0.1
		20	14.2 ± 1.2	7.0 ± 0.7	10.6 ± 0.2
	120	10	14.2 ± 0.1	5.7 ± 0.0	13.8 ± 0.5
		15	**17.7 ± 0.8**	5.9 ± 0.1	9.0 ± 0.1
		20	17.3 ± 0.5	8.0 ± 0.0	10.5 ± 1.0
1.5	100	10	14.2 ± 0.7	5.6 ± 0.2	13.8 ± 0.4
		15	14.4 ± 0.0	4.9 ± 0.3	12.0 ± 0.8
		20	14.5 ± 0.1	6.8 ± 0.4	**15.4 ± 1.0**
	110	10	13.0 ± 0.1	5.4 ± 0.1	12.2 ± 0.3
		15	12.2 ± 0.3	5.9 ± 0.1	13.6 ± 0.1
		20	8.5 ± 0.6	5.9 ± 1.0	11.9 ± 0.1
	120	10	16.9 ± 0.8	8.6 ± 0.4	10.9 ± 0.2
		15	17.2 ± 0.1	7.7 ± 0.2	12.8 ± 0.1
		20	15.1 ± 0.0	6.4 ± 0.1	12.1 ± 0.1

Data represented in the above table as ± SD (standard deviation, n=3)

Similarly, Satlewal *et al.* (2018b) reported that the pretreatment at optimized conditions yield higher sugar concentration with minor amount of fermentation inhibitors. Hence, detoxification process would not be needed and the slurry can be used for enzymatic

hydrolysis as well as fermentation. However, the amount of sugar released in both chemical treatments was dependent on the nature of the substrate used for analysis. Similar results were reported in a study by Zhao *et al.* (2012).

In one of the study, the rice straw was pretreated by acid at optimized conditions (2M H_2SO_4, 90 °C and 60 min) followed by enzymatic hydrolysis yielded 11.4 g/L glucose (Aditiya *et al.* 2015). A study by Feher *et al.* (2017) applied acid hydrolysis on corn stover, and high xylose yield was observed in both hydrochloric acid and sulfuric acid treatments. Moreover, total sugar yield was recorded after recycling of sulfuric acid hydrolysate (at 140 °C, 40 min and 7% (w/w) dry matter), and 11.6 g/L glucose, 47 g/L xylose, and 7.3 g/L arabinose was obtained. Similarly, in one of the previous studies, aqueous ammonia (27% w/w) was used for pretreatment of rice straw before enzymatic hydrolysis at two temperatures (60 °C and room temperature) for 3 days. The range of glucose yield for 3 to 7 days at 60 °C and room temperature was 1.24 to 1.65 g/L and 1.43 to 1.72 g/L, respectively (Phitsuwan *et al.* 2017).

Cultivation of the *C. vulgaris* and the Carbohydrate Accumulation

The overall ethanol production from the microalgae is shown in Fig. 1. The *C. vulgaris* was cultured in the BG-11 medium and the nitrogen deficient medium for 7 d under 24 h light. The yield of the freeze-dried *C. vulgaris* biomass that was cultivated in the BG-11 medium was 1.5 g/L ± 0.1 g/L *versus* 1.0 g/L ± 0.0 g/L for the nitrogen deficient conditions (Fig. 2). The *C. vulgaris* was grown in the nitrogen deficient medium to obtain a carbohydrate enriched biomass as a raw material to produce alcohol. The *C. vulgaris* had a biomass productivity of 231 mg/L/day ± 0.9 mg/L/day and a carbohydrate content of 33% ± 0.2% in the BG-11 medium before the employment of the stress condition. However, in the nitrogen deficient condition, the carbohydrate contents increased up to 51% ± 2.1%. These results were also in line with the results from Behrens *et al.* (1989). However, the protein content decreased from 41% ± 0.7% to 12% ± 1.0%. This may have been due to the nitrogen used for the synthesis of the cell structures and enzymes. Therefore, the fixed carbon dioxide is converted into lipids or carbohydrates rather than protein (Richardson *et al.* 1969; Rodolfi *et al.* 2009). Some studies have demonstrated that there is also a competition between the synthesis of starch and lipid under deficient conditions (Rismani-Yazdi *et al.* 2011; Siaut *et al.* 2011).

Table 4. Composition of the Microalgae Grown in the BG-11 Medium and the Nitrogen Deficient Medium

Parameters	BG-11 Medium	Nutrient Deficient Medium
Biomass Concentration (g/L)	1.5 ± 0.1	1.0 ± 0.0
Biomass Productivity (mg/L/day)	231 ± 0.9	135 ± 0.8
Carbohydrates (% dry wt)	33 ± 0.2	51 ± 2.1
Proteins (% dry wt)	41 ± 0.7	12 ± 1.0
Lipids (% dry wt)	20 ± 0.2	28 ± 0.2

Data represented in the above table as ± SD (standard deviation, n=3)

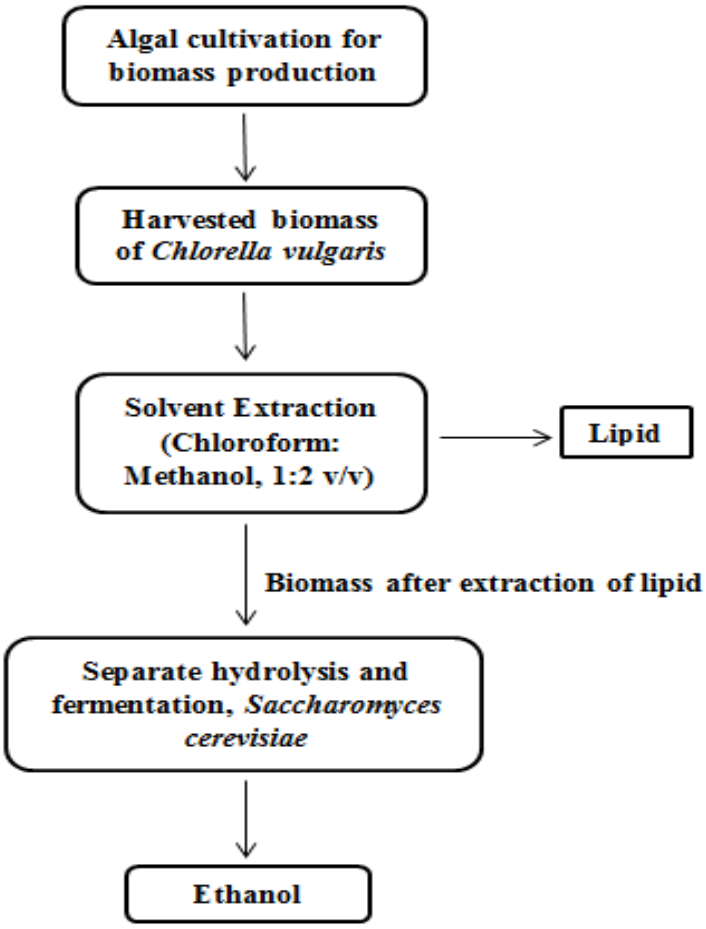

Fig. 1. Integrated ethanol production process from *C. vulgaris*

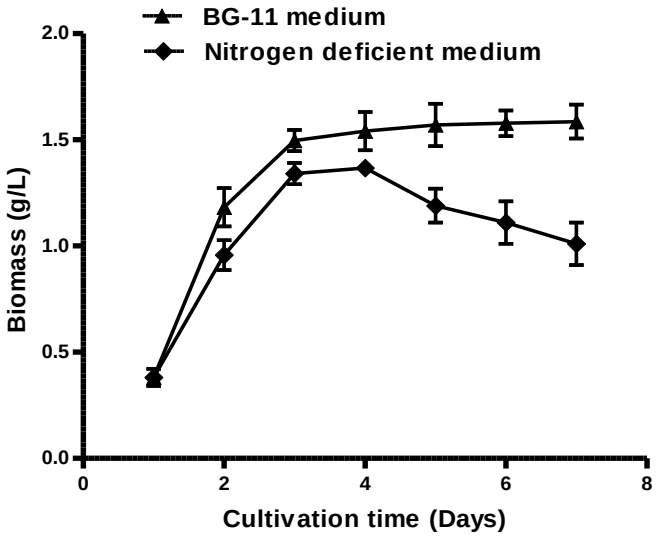

Fig. 2. Growth curve of *C. vulgaris*

Acid Hydrolysis of the Algal Biomass for Sugar Production

The *C. vulgaris* biomass was obtained after centrifugation at 5,000 rpm for 10 min. The calculated biomass concentration was 1 g/L in the nitrogen deficient medium. To achieve a higher biomass concentration and productivity, the mixotrophic cultivation condition was preferred (Kim *et al.* 2013; Praveenkumar *et al.* 2014). The lipids were extracted by the solvent extraction method to produce biodiesel as a side product. The remaining biomass was subjected to the acid hydrolysis treatment for ethanol production. The solvents remove the lipids and the chlorophyll, and the color of the biomass appeared pale yellow with a high carbohydrate content. The GC analysis showed that the extracted lipids from the *C. vulgaris* biomass have the potential to produce biodiesel. The summary of the major fatty acids is shown in Table 5 which shows that its oil can be used for biodiesel production. The main components were palmitic acid, oleic acid, and linoleic acid, which had compositions of 33.1%, 21.6%, and 8.1%, respectively.

Table 5. Major Fatty Acids of the Extracted Lipids from *C. vulgaris*

Common Names	Empirical Formula	Fatty Acid Composition (%)
Stearic Acid	C18:0	7.3
Oleic Acid	C18:1	21.6
Palmitic Acid	C16:0	33.1
Palmitoleic Acid	C16:1	5.7
Linoleic Acid	C18:2	8.1
Linolenic Acid	C18:3	1.4
Other Acids	-	22.8

Data represented in the above table as ± SD (standard deviation, n=3)

After the lipid extraction process, the freeze-dried biomass was subjected to acid hydrolysis to obtain monomeric sugars. Dilute acid hydrolysis is an efficient method to produce more than 90% sugar using a low solid loading concentration (Ho *et al.* 2013). The sample was hydrolyzed using 1% (v/v) H_2SO_4 and 8% (w/v) solid loading. Harsh hydrolysis conditions were not applied in this study to prevent the formation of fermentation inhibitors. These conditions generated a higher glucose concentration of 32 g/L. One study reported that the optimum temperature for the pretreatment of wastewater algae is 80 °C to 90 °C (Castro *et al.* 2015). In other studies, a higher temperature of 110 °C to 130 °C was used for the acid hydrolysis, which ultimately affects the cost of heat energy (Wang *et al.* 2011; Yazdani *et al.* 2011; Setyaningsih *et al.* 2012). This is because the sterilization process usually requires a temperature of 120 °C. However, the effect of the T_r is also dependent on the acid concentration (Yazdani *et al.* 2011). The acid concentration is also directly dependent on the amount of sugar obtained after the acid hydrolysis.

Separate Hydrolysis and Fermentation

The agricultural waste samples and *C. vulgaris* had the highest sugar contents at acidic conditions as compared to alkaline pretreatment conditions, so they were selected as the fermentation substrates. The conversion of hydrolysed samples into alcoholic fuel was carried out by ethanol producing strain *S. cerevisiae* and butanol producing strain *C.*

acetobutylicum individually. The productivity of ethanol and butanol as well as yield from feedstock hydrolysates is shown in Table 6. The *C. vulgaris* and wheat straw among lignocellulosic materials contain glucose contents of 32 g/L and 20.6 g/L, respectively. After 48 h of fermentation procedure, the ethanol concentration in all of the feedstocks ranged from 5.8 g/L to 14 g/L, whereas the butanol concentration was from 5.0 g/L to 11.8 g/L. However, among the agrowastes, the wheat straw produced the highest amount of ethanol concentration (9.5 g/L_± 0.5 g/L) and ethanol yield of 93.7% and butanol concentration (7.4 g/L ± 0.1 g/L) and butanol yield of 91.3%. The maximum ethanol concentration 14 g/L ± 0.3 g/L and ethanol yield (89.1%) as well as butanol concentration and yield of 11.8 g/L and 94.4% respectively were recorded in *C. vulgaris*. The higher carbohydrates and glucose contents in *C. vulgaris* makes it a promising substrate for alcohol production.

Various recent studies have reported the fermentation of both microalgae and macroalgae biomass. Only a few of the studies used lipid extracted microalgae as a fermentation substrate for alcohol production. In a recent study, lipids were extracted from *Chlorella sorokiniana via* solvent extraction, and the enzymatic hydrolysis method was employed to produce butanol (Cheng *et al.* 2015). Similarly, the conversion of *C. vulgaris* biomass into butanol by fermentation using *C. acetobutylicum* was studied by Wang *et al.* (2016), and a higher butanol production (13.1 g/L) was reported. A study reported on the acid hydrolysis of dried microalgae biomass at optimized conditions yield 3.74 g/L butanol by fermentation by *Clostridium saccharoperbutylacetonicum* (Castro *et al.* 2015). Toquero and Bolado (2014) reported that alkaline pretreated wheat straw hydrolysate yields glucose (31.82 g/L) and xylose (13.75 g/L) and after fermentation, 17.37 g/L ethanol was produced.

To produce alcoholic fuels (butanol and ethanol) from lignocellulosic and microalgae feedstock, various technological steps are required. To accomplish the cost-effective production of biofuels, it is important to properly adjust all units of a system. There are popular cases of the production of biomass-based fuels in developed countries that may be a good reference for developing countries. The cost of fuels can be decreased when they are produced at an industrial scale. Innovating and cost-effective production processes can lead to the production of plant-based biofuels, which are currently underutilized (Tao *et al.* 2014).

Table 6. Fermentation of WS, RS, CS, and CV with S. *cerevisiae* (Ethanol Production) and C. *acetobutylicum* (Butanol Production)

Substrates	G_{TC} (g/L)	TY_E (g/L)	AY_E (g/L)	$F_{Effi\ (E)}$ (%)	TY_B (g/L)	AY_B (g/L)	$F_{Effi\ (B)}$ (%)
WS	20.6	10.13	9.5	93.7	8.1	7.4	91.3
RS	15.4	7.37	5.8	78.6	5.9	5.0	84.7
CS	17.8	8.29	7.7	92.8	6.7	5.2	77.7
CV	32.0	15.7	14	89.1	12.5	11.8	94.4

Notes: G_{TC} = Total concentration of Glucose, TY_E = Theoretical yield of Ethanol, AY_E = Actual yield of Ethanol, $F_{Effi\ (E)}$ (%) = Fermentation Efficiency of Ethanol, TY_B = Theoretical yield of Butanol, AY_B = Actual yield of Butanol, and $F_{Effi\ (B)}$ (%) = Fermentation Efficiency of Butanol

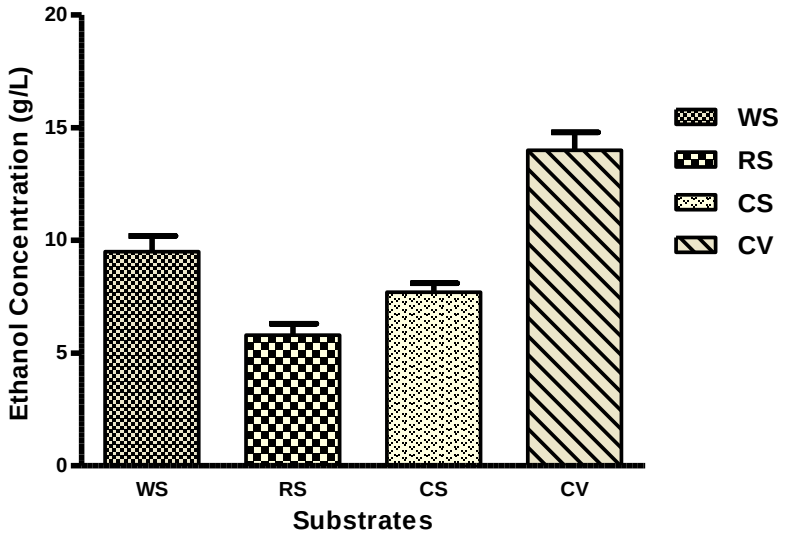

Fig. 3. The ethanol production from the agrowaste biomass samples and *C. vulgaris* by *S. cerevisiae* with the H_2SO_4 pretreated samples; WS = wheat straw, RS = rice straw, CS = corn stover, and CV = *C. vulgaris*

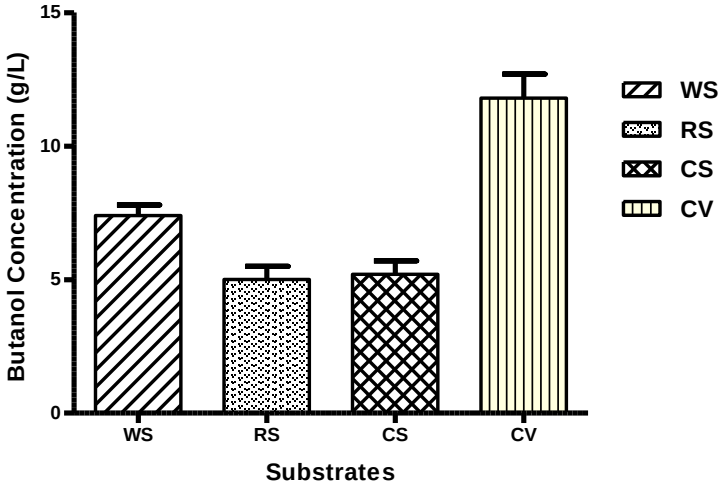

Fig. 4. The butanol production from the agrowaste biomass samples and *C. vulgaris* by *C. acetobutylicum* with the H_2SO_4 pretreated samples; WS = wheat straw, RS = rice straw, CS = corn stover, and CV = *C. vulgaris*

CONCLUSIONS

1. The lignocellulosic biomasses wheat straw, rice straw, and corn stover (WS, RS, and CS) were effectively hydrolyzed by acidic as well as basic pretreatment in comparison followed by an enzymatic hydrolysis. It was observed that acidic pretreatment was far better and yields high glucose contents and wheat straw is showing the higher glucose concentration of 20.6 g/L under optimized cultivation conditions.
2. Cultivation of *C. vulgaris* in nutrient deficient medium exhibited an increased carbohydrate concentration (51% ± 2.1%) and glucose yield of 32 g/L and its conversion into alcohol (ethanol and butanol) is most promising option.

3. The fatty acid profile of extracted lipid from *C. vulgaris* biomass showed its co-production of biodiesel as a side product.
4. Higher concentration of ethanol was achieved by *C. vulgaris* (14 g/L) followed by wheat straw (9.5) by separate hydrolysis and fermentation using *S. cerevisiae.*
5. *C. vulgaris* also exhibit a high butanol contents (11.8 g/L) and yield of 94.4% which concludes that it can be used for commercial production of biofuel.

ACKNOWLEDGMENTS

The authors are thankful for the support of the Higher Education Commission (HEC) of Pakistan for providing funds to conduct the "Production of Butanol and Ethanol from Agriculture and Waste" research project.

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Article submitted: May 2, 2019; Peer review completed: July 29, 2019; Revised version received and accepted: August 21, 2019; Published: August 27, 2019.
DOI: 10.15376/biores.14.4.8135-8154