INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0826/202x/00–0–000–000
DOI: 10.17957/IJAB/15.1582
http://www.fspublishers.org

Full Length Article

Evaluation of Fungal and Sonication Pretreatments to Improve Saccharification Yield of *Arundo donax*

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Received 28 May 2020; Accepted 14 July 2020; Published _____

Abstract

Arundo donax L., as energy crops, is an attractive, inexpensive alternate lignocellulosic feedstock, which provides very responsible use of natural resources by not compromising food crops and mitigating fossil fuels shortage. The objective of current research was to evaluate fungal and sonication pretreatments to investigate availability of fermentable sugars from *A. donax* biomass to improve preexisting physico-chemical approaches for bioethanol production. *Trichoderma koningii* and *Aspergillus niger*, two fungal strains were employed to optimize the release of fermentable sugars. Effect of sonication pretreatment at 50 Hz frequency for 15, 30, 45 and 60 min was evaluated prior to enzymatic hydrolysis. Structural analysis with scanning electron microscopy (SEM) exposed discernible surface morphology with rough, disintegrated and porous surfaces of pretreated biomass. Highest glucose yield after14 days of pretreatments at 72 h of enzymatic hydrolysis by *T. koningii* and *A. niger* reached 237.7 ± 0.7 and 214.8 ± 0.8 mg g^{-1} respectively. Sonication for 60 min followed by 45, 30 and 15 min pretreated biomass showed highest reducing sugar yield (204.5 ± 0.4, 161.4 ± 0.2,152.6 ± 0.4 and 76.1 ± 0.2 mg g^{-1}), respectively significantly much higher than untreated (67.1 ± 0.4 mg g^{-1}) at 72 h of enzymatic hydrolysis. Our results implicate an optimal harvest time for achieving highest yield of fermentable sugars after *T. koningii* pretreatment. © 2020 Friends Science Publishers

Keywords: *Arundo donax* L.; Fungal saccharification; Sonication; SEM

Introduction

Arundo donax L. a nonfood lignocellulose biomass does not compete with other feed and food crops considered as potential alternate renewable crop yielding high proportions of transportation fuel by employing cost effective and operative conversion methods. This species is considered as a valuable source of biomass feedstock due to persistent yield, malleability to marginal environments with low input requirements among other lignocellulosic biomass (Amaducci and Perego 2015). The survivability to drought resistance and salt tolerance make it suitable energy crop which can be cultivated in low quality irrigation waters (Accardi *et al.* 2015).

A. donax a member of Poaceae family represents numerous remarkable features as potential dedicated biomass energy crop (Corno *et al.* 2014; Lemões *et al.* 2018) and acquainted by number of common names including, bamboo reed, bamboo, false bamboo, *Arundo* grass, reed grass, giant reed, giant reed grass, giant Danube reed, bamboo cane, giant cane, Canne-de Provence, wild cane, Spainsh cane and *A. donax* cane. In Pakistan it is commonly known by "Nurr", "Nurro" or "Nurru," (Maria *et al.* 2013) and saroot. This plant flourishes abundantly and spontaneously in southern Europe and several subtropical temperate regions. *A. donax* was habituated from Asia through Middle East to whole Mediterranean basin during prehistory (Corno *et al.* 2014). Widely divergent archeological and historical evidences witness the origin of *A. donax* from Asia, North Africa, North and South America, South Europe, Middle East moreover in Australia (Saikia *et al.* 2015). In its native range, *A. donax* is abundant in Pakistan and India ascending to 2500 m elevations in Himalayas and spreads throughout China and South-East Asia.

A. donax is an erect, tall, sterile rhizomatous (Scordia *et al.* 2011) perennial C3 grass grows well up to 9 m (Saikia

et al. 2015) in dense stands (Krička *et al.* 2017). Due to fast growth of *A. donax* in water, it usually falls in category of emergent aquatic plant (Angelini *et al.* 2009). The potential yield of *A. donax* dry biomass is 29–46 tons ha^{-1} $year^{-1}$ that entirely depends on geographical positions and climatic conditions (Pari *et al.* 2015) besides moisture content, density and time of cultivation (Krička *et al.* 2017).

Various approaches are also evolving to utilize crop lands that are not fit for traditional food crops better for growing energy crops to avoid competition for land between nonfood and food crops (Giacobbe *et al.* 2016). Keeping in view, the cost effectiveness, lignocellulosic feedstocks have several advantages over other agricultural feedstocks like; potatoes, cornstarch, sugarcane juice in addition to easy production with low cost unlike other food crops. *A. donax* can also be considered a good candidate to supplement or replace maize, sorghum and other energy food crops in particular to produce green energy (Pilu *et al.* 2013).

The lignocellulosic biomass structure is highly complex polymer, primarily composed of cellulose, hemicelluloses and lignin which is not directly accessible for microbial or enzymatic degradation (Ghorbani *et al.* 2015). This makes it a major limitation for efficient bioethanol production. Prior to the conversion from biomass to bioethanol, effective pretreatment approach is a prerequisite (Scordia *et al.* 2011) in order to break lignin and hemicellulose making cellulose available to hydrolyzing enzymes for the release of sugar monomers that can finally be converted into ethanol or any other valuable products (Huang *et al.* 2015).

The pretreatment processes are used to increase carbohydrate degradability and porosity, remove lignin and preserve hemicellulosic constituents (Gupta and Lee 2010; Chiaramonti *et al.* 2012). The pretreatment and conversion of lignocellulosic biomass into hydrolysable constituents by alkali, acid, microbes and commercial enzymes is very challenging. Alkaline (NaOH) pretreatment studies reported for *Miscanthus*, wheat straw and cotton stalk show effects on delignification and ultimately on enzymatic hydrolysis yield. Some contents of hemicelluloses and cellulose were also degraded and removed from biomass feedstocks by the action of hydroxide ions in addition to delignification during alkaline pretreatment (Cheng *et al.* 2010).

Ultrasound, a sound wave, through agitation and cavitation in liquid can produce energy and has great potential to damage the surface structure of biomass. Ultrasound mainly applied to supplement pretreatment of various lignocellulosic biomass with different reaction solutions (Wang *et al.* 2016). However, for both bioethanol and biogas production, a higher susceptibility of biomass to pretreatments will allow the use of more environmentally friendly processes, by lowering pollution and energy costs.

Biological pretreatments are based on using microorganisms capable of degrading cellulose, lignin and hemicellulose. Cellulose fraction is perhaps the most resistant component to biological attack. White, soft and brown rot fungi are mainly used to pretreat lignocellulosic feedstocks and enhance the enzymatic hydrolysis yield (Anwar *et al.* 2019). Brown rots fungi mostly degrade cellulose, whereas soft and white rots mainly involved in both lignin and cellulose degradation. White rot fungi are considered as among the most effective basidiomycetes for biodegradation of lignocellulosic biomass (Sun and Cheng 2002). The low energy consumption, ecofriendly, cost effective, absence of chemicals and inhibitory compounds, simple and lesser requirements are imperative aspects of microbial pretreatment which attracts attention of scientists and researchers (Chiaramonti *et al.* 2012). Fungi can efficiently produce ligninolytic enzymes, which play a key part in biological pretreatments. Fungi by biodegrading lignin improve availability of enzymes to the cellulose in lignocellulosic biomass structure. Consequently, modified biomass is more vulnerable to enzymatic degradation and digestion (Ghorbani *et al.* 2015). Moisture content, particle size, pretreatment time and temperature significantly affect degradation of lignin and enzymatic saccharification yield (Kumari and Singh 2018).

Although an enormous amount of literature is available with respect to second generation biomass, but no work has been reported so far on the potential of *Arundo* biomass after being pretreated comparatively with fungus and sonication. The current study was designed with the goal to investigate effective approaches amongst different fungal and sonication pretreatments in order to improve bio-delignification, comparative importance of each pretreatment on rate of fermentable sugars and efficacy of conversion by enzymatic hydrolysis of *A. donax*. Comparative evaluation of different pretreatment approaches and their impact on saccharification yield from *A. donax* was the main objective of current study.

Materials and Methods

Collection and preparation of *A. donax*

The sampling of *A. donax* was carried out in October from Kallar Kahar Lake, a brackish lake with geographical coordinates, Latitude: 32° 47' 0" North; Longitude: 72° 42' 0" East (Ahmad and Erum 2012) situated in Jhelum respectively, Punjab, Pakistan. In current research plant culms of *A. donax* were obtained for estimation of reducing sugar and bioethanol production. Physiological parameters were measured then finely cut from internode 2 with sharp sterile plant cutter. Three biological repeats were collected. *A. donax* biomass was brought to lab, cleaned, weighed and the leaves were removed. The *Arundo* biomass was initially dried at 45°C for 72 h (Zakir *et al.* 2016) and stored in airtight bags.

Fungal strains and growth conditions

The fungal strains; *Trichoderma koningii* and *Aspergillus*

niger were provided by the Department of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan. The strains were aseptically cultured for 7 days at 30°C on potato dextrose agar (PDA) plates. Fungal strains were maintained on sterile PDA plates and preserved at 4°C (Ghorbani *et al.* 2015). The freshly grown mycelium were further inoculated into 250 mL Erlenmeyer flasks in 30 mL of PD broth growth medium at pH 5.6 and incubated at 30°C with 180 rpm for 7 days for fungal pretreatments.

Reagents and enzymes

The reagents used in experiments were sodium acetate, glucose, citrate buffer, glacial acetic acid, antibiotics i.e., tetracycline hydrochloride and cyclohexamide and all the chemicals used throughout current experimental study were procured from Sigma-Aldrich (Beijing, China), cellulase of *T. reesei* from Shanghai Boao Biotech. Corp., Shanghai, China were of highest purity and analytical grade. Water was purified using (Master-D series) high performance ultra-pure water system.

Pretreatment of *Arundo* biomass

In order to investigate higher yield of reducing sugar and release efficiency; physical, fungal and sonication pretreatments were carried out. Untreated biomass was taken as control. All experiments were carried out in triplicates.

Physical fragmentation: The internodes 2–5 of each collected plant were combined for making a composite biomass then chipped and pulverized in micro soil plant disintegrator crusher pulverizer grinding mill (FT102). For achieving uniform particle size, the ground biomass was screened through 20 mesh sieves to attain homogenous particle size (850 µm) for efficient pretreatment of *Arundo* biomass. The ground biomass was stored in sterile airtight polybags at room temperature under dry conditions until use for further analysis and pretreatments (Giacobbe *et al.* 2016; Silverstein *et al.* 2007).

Bio-delignification: For bio-delignification experiments the respective sterile aqueous culture medium containing 5 g L^{-1} yeast extract, 15 g L^{-1} glucose and 15 g L^{-1} peptone were prepared aseptically. The aqueous solution was then enriched by copper ($CuSO_4 \cdot H_2O$), manganese ($MnSO_4 \cdot H_2O$) and zinc ($ZnSO_4 \cdot 7H_2O$) ions with final concentrations equal to 2.5 µM, 0.1 mM and 5 µM, respectively pH of the prepared solution adjusted at 4 ± 0.05. Fungal pretreatment carried out separately by addition of 100 mL prepared culture medium to 4 g of *Arundo* dry biomass in 500 mL Erlenmeyer flasks. Following sterilization, each flask was then inoculated by two plugs of 10 mm diameter with 5 days fresh grown PDA fungal culture medium (*T. koningii* and *A. niger*), capped with hydrophobic sterile cotton plugs and incubated in an orbital shaker at 30°C, 160 rpm (Ghorbani *et al.* 2015) up to 14 days. The biopretreated and untreated *A. donax* samples were withdrawn from triplicate flasks of each fungal culture; *T. koningii* and *A. niger* after 7 and 14 days. Pretreated and untreated samples were washed with double distilled water (50 mL) at 28°C at 180 rpm for 1 h, then vacuum filtrated through ceramic Buchner funnel (SHB III, TOPTION) with filter paper lining to separate liquid and solid and remove most of water-soluble components. Solid fraction was extensively washed with distilled water until neutral pH attained followed by last washing with 50 mM citrate buffer (pH; 5) that subsequently used in enzymatic hydrolysis (Carvalho *et al.* 2013; Amezcua-Allieri *et al.* 2017), samples were vacuum filtered through Buchner funnel and oven dried at 60°C for 24 h to a constant weight (Mishra *et al.* 2014; Ghorbani *et al.* 2015; Wang *et al.* 2016; Zakir *et al.* 2016). After cooling down, pretreated dried residues were kept in desiccator, collected and stored in zip-lock bags at room temperature for enzymatic hydrolysis. Untreated (non-inoculated) biomass samples were taken as control, incubated and further treated under same conditions.

Sonication: *A. donax* (1 g DM) ground biomass was mixed in 100 mL of distilled water and subjected to sonication at frequency of 50 Hz for 15, 30, 45 and 60 min. The slurry was vacuumed filtered, washed with distilled water (H_2O) and dried at 70°C for 24 h (Mishra *et al.* 2014). The solid fraction of *Arundo* biomass was then proceeded for enzymatic hydrolysis.

Enzymatic hydrolysis (EH)

Enzymatic saccharification of fungal and sonication pretreated biomass as well as their respective control samples of *A. donax* was carried out using commercial cellulase derived from *T. reesei* (≥ 700 units) containing 60 FPU g^{-1} (Filter Paper Unit per gram) enzyme activity. The 30 FPU g^{-1} of enzyme was added to dried biomass (Wu *et al.* 2016). Enzymatic hydrolysis was performed in triplicate using 250 mL sterile glass reactors, each containing 50 mL of sodium acetate buffer: 50 mM L^{-1}; pH 5 at room temperature which was prepared in autoclaved distilled water. The residual pretreated dry biomass 1g was mixed with acetate buffer resulting concentration of substrate 2% (w/v). Enzymatic reaction proved more effective when diluted with buffer as compared to distilled water (Zakir *et al.* 2016). Each enzymatic hydrolysis mixture containing 40 µg mL^{-1} tetracycline (Cheng *et al.* 2010) and 30 µg mL^{-1} cyclohexamide was incubated at 48°C for 72 h with 120 rpm in an orbital incubator shaker (MaxQ 8000, Thermo Fisher). Adding cyclohexamide inhibits DNA translation of the eukaryotic cells to inhibit cell growth which ultimately leads to death of cell. The main target of using cyclohexamide and tetracycline hydrochloride was the inhibition of microbial growth that affects pH during enzymatic hydrolysis process and enzymatic activity (Silverstein *et al.* 2007; Wang *et al.* 2018).

Samples of 2 mL were withdrawn after 24, 48 and 72 h of enzymatic saccharification to evaluate glucose concentration. Hydrolysates were heated for 10 min in

boiling water to stop enzymatic reaction (Martin-Sampedro *et al.* 2017), cooled at room temperature and separated by centrifugation at 10,000 rpm for 10 min. Then collected the supernatant and filtered through 0.2 µm nylon syringe filters (Wang *et al.* 2018) and stored in refrigerator at -20^{o}C for further analysis. Consequently, quantity of reducing sugars was measured by using glucose calibration curve (Amezcua-Allieri *et al.* 2017) using (Beckman DU640 UV/Vis) spectrophotometer at 540 nm. Reducing sugar (glucose) concentration was measured by 3, 5-dinitrosalicylic acid (DNS) by Miller (1959). The values presented in the results were means of triplicates with the values of standard deviation and calculated in mg of reducing sugar per g dry weight of *A. donax* biomass by following equation:

$$\text{Reducing sugar yield (mg g}^{-1}\text{ dry biomass)} = (\rho \times \nu) / m$$

where "ρ" is reducing sugars concentration (mg mL^{-1}) from hydrolysate, "ν" total volume hydrolyzed (mL), "*m"* initial dry weight (g) of *Arundo* biomass. The values were expressed in mg g^{-1} basis.

Physico-chemical characteristics of *Arundo* biomass

The moisture, volatile matter and ash of well dried raw *A. donax* of October were analyzed according to standard protocols; ASTM D 3174, ASTM D 3173 and ASTM D 3175, respectively on the dry weight basis. Fixed carbon content of biomass was calculated by the difference. The fixed carbon content is the value of difference from 100% biomass to ash, moisture and volatile matter percent on dry weight basis (Saikia *et al.* 2015). While Cellulose, hemicelluloses and lignin content of raw *Arundo* biomass were determined by acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) methods (Omar *et al.* 2011; Saikia *et al.* 2015) with some modifications. The elemental analysis carbon, hydrogen, nitrogen and sulphur of well dried raw *Arundo* biomass was analyzed by automatic CHNS analyzer (Vario EL cube). The content of oxygen was measured by calculating the difference of O (%) = 100 (%) – C (%) – H (%) – N (%) – S (%) (Licursi *et al.* 2015). The values reported in results are the mean ± standard deviation of three replicates.

Scanning electron microscopy (SEM)

The characteristics of surface morphology of *A. donax* biomass were scanned by using SEM (HITACHI-S-3400N), current; 30 mA, voltage; 15 kV and distance; 14.3 mm. Untreated and pretreated *A. donax* biomass samples were dried in oven at 50^{o}C for 24 h (Wang *et al.* 2018) for removing moisture content. Dried *Arundo* biomass (1 mg) was examined and photographed by using SEM to investigate surface morphology of each sample both in degraded (pretreated) and intact (untreated) biomass.

Statistical analysis

To verify differences regarding untreated and pretreated *Arundo* biomass by an analysis of variance; univariate one-way analysis of variance (ANOVA), following post hoc multiple comparison Tukey and Duncan's test by using Statistical Product and Services Solutions (SPSS) version 23 at statistical significance level of 0.05. A multifactorial design was employed comprising dependent variables; fungal strains *T. ressei* and *A. niger*, duration of pretreatment 7, 14 days and harvest time, sonication time and independent variables was glucose yield.

Results

Characterization of *Arundo* biomass

The proximate analysis reveals fixed carbon (20.8%), volatile matter (71% ± 0.76), ash (4% ± 0.01) and moisture content (4.2% ± 0.01%) whereas compositional analysis showed cellulose (24%), hemicellulose (30%) lignin (26.8%) and other components (16.9%) and elemental analysis demonstrated nitrogen (0.31 ± 0.02%), sulfur (0.09% ± 0.004), carbon (45.1% ± 0.01), hydrogen (5.9% ± 0.03) and oxygen (48.69%) were found in *Arundo* biomass on percent dry weight basis are presented in Table 1. Based on the results of elemental analysis, H:C ratio computed is 0.13 and O:C ratio is 1.07.

SEM analysis

The SEM images of degraded and non-degraded biomass indicated that pretreated biomass exposed many discernible surface morphologies with porous and rough surfaces might be due to removal of lignin and hemicelluloses after pretreatments compared to untreated biomass (Fig. 1). Untreated *Arundo* biomass (Fig. 1a) had intact, compact and smooth surfaces. Fig. 1b revealed layering, scaling and visible abrasions of fibers due to degradation of lignin and hemicelluloses after *T. koningii* pretreatment. *A. niger* pretreated biomass (Fig. 1c) resulted in mild scaling and layering as compared to Fig. 1b which may possibly be owing to partial decomposition of hemicelluloses. However, significant cavitation and shock effect of sonication enhanced removal of lignin beside considerably increased degradation of hemicelluloses (Fig. 1d–g). The surface of *Arundo* biomass revealed few sunken areas beside layering and scaling (Fig. 1d), also with erosion troughs and clear cracks (Fig. 1e). Also, there was disintegration of upper layer (Fig. 1f) as well as scaling and cavitation in *A. donax* biomass was noted (Fig. 1g). Among all pretreated biomass highest disintegration of surface morphology could be observed in *Trichoderma* pretreated biomass rendering it advantageous for enzyme accessibility and processivity. *Trichoderma* pretreated biomass was more accessible for enzyme among all other pretreated biomass. Among all

Table 1: Characteristics of raw *A. donax* biomass% dry weight

Characteristics	%age
Proximate	
Moisture content	4.20 ± 0.01
Ash	4.00 ± 0.01
Volatile matter	71.00 ± 0.76
Fixed carbon[a]	20.80
Ultimate	
Carbon	45.10 ± 0.01
Hydrogen	5.90 ± 0.03
Nitrogen	0.31 ± 0.02
Sulfur	0.09 ± 0.004
Oxygen[b]	48.69
Compositional	
Cellulose	24.00
Hemicellulose	30.00
Lignin	26.80
Others[c]	16.90

Results are presented as means of three repeats with the values of standard deviations. **(a)** Remaining organic matter after biomass volatile matter and moisture have been driven off; **(b)** Oxygen content was calculated by difference. O (%) = 100 (%) – C (%) – H (%) – N (%); **(c)** Calculated as the difference between 100% and the sum of the composition of four components i.e. ash, cellulose, hemicellulose and lignin

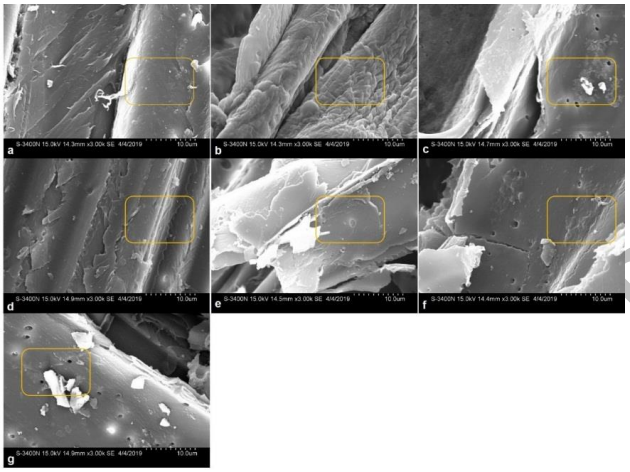

Fig. 1: Scanning electron microscopy (SEM) images of untreated and pretreated *A. donax* biomass at 3k x magnification with 10 µm scale bar. a: Untreated, b: *T. koningii*, c: *A. niger*, d: Sonication 15 min (S15), e: Sonication 30 min (S30), f: Sonication 45 min (S45), g: Sonication 60 min (S60)

pretreated biomass samples, morphological structure of *T. koningii* pretreated biomass was highly destroyed and disintegrated, making it more advantageous for saccharification. This observation was in accordance with our results of *T. koningii* having maximum yield of glucose after enzymatic hydrolysis (Fig. 2).

Effect of fungal and sonication pretreatments on enzymatic saccharification

Fig. 2a showed the effect of incubation for 7 and 14 days after pretreatment with two fungal strains *T. koningii* and *A. niger* along with untreated *Arundo* biomass and the production of reducing sugars (glucose) at 24, 48 and 72 h of enzymatic hydrolysis process. The cellulose digestibility was substantially enhanced by *T. koningii* resulting in maximum fermentable sugars release. The optimum glucose 237.7 ± 0.7 mg g^{-1} dry solids was released after 14 days of *T. koningii* pretreatment at 72 h of enzymatic hydrolysis and significantly higher than untreated *Arundo* biomass 106.6 ± 0.3 mg g^{-1} solid dry biomass (p value <0.05) while *A. niger* pretreated biomass liberated 214.8 ± 0.8 mg g^{-1} solid dry biomass of glucose with same conditions. All pretreated biomass exhibited similar trend of increased glucose yield by increasing duration of enzymatic hydrolysis. The prolonged duration of pretreatment and enzymatic hydrolysis favored efficient generation of glucose. Among enzymatic saccharification of each pretreatment 72 h released maximum glucose yield followed by 48 and 24 of the reaction which is statistically significantly different (Table 2). Data showed that with sonication time ranging from 15–60 min at 15 min interval on glucose harvest the digestibility of cellulose content and glucose yield was significantly enhanced by increased duration of sonication (Fig. 2b). It was found that sonication for 60 min liberated more reducing sugars (glucose) followed by 45, 30 and 15 min (Table 3).

Discussion

Proximate, ultimate and compositional analyses reveal the optimal values of their respective compositions in *A. donax* (Table 1). The moisture content (4.2 ± 0.01%) of *A. donax* in current study is in accordance to the values of moisture content and dry matter for *A. donax* reported in previous studies (Mejdi *et al.* 2010; Natalia and Adam 2011). Moisture content of the crop depends on many factors and remains relatively low, rendering the dry biomass high. Moisture content is of paramount importance since lower the moisture content of biomass would decrease the tendency of decomposition and save energy (Sánchez *et al.* 2019), in addition, less energy would be required for size reduction (Ani 2015) which would reduce pretreatment time and decrease the cost of pretreatment (Quintero *et al.* 2011); Low moisture content would be more favorable to stop anaerobic microbial degradation thus also permit safe long term storage of the biomass (Rentizelas 2016). Lower ash content (4 ± 0.01%) in current study is a favorable characteristic regarding biofuel production and is in line with the previous reports (Vernersson *et al.* 2002; Krička *et al.* 2017). The ash content is one of the most remarkable characteristics of LB Biomass, as it comprises inorganic matter and minerals, being an integral part of biomass, it affects the combustion rate. Lower ash content is optimally most conducive to a favorable outcome with respect to biofuel production (Singh 2019). A high volatile matter (71 ± 0.76%) in *A. donax* biomass found in current study is another good attribute for biofuel production (McKendry 2002), which is also consistent with the results (71.3%) reported by Vernersson *et al.* (2002). The fixed carbon content and volatile matter affects the biological conversion

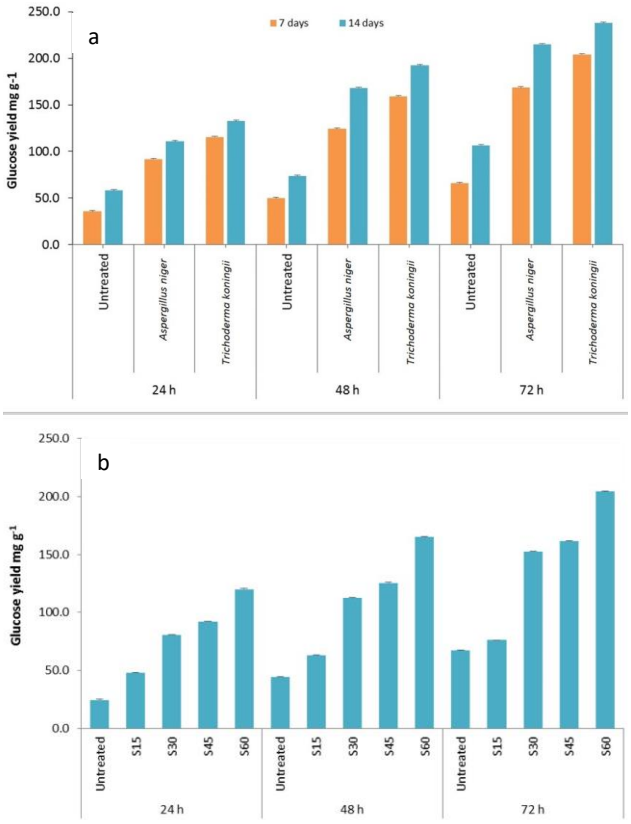

Fig. 2: (a) Effect of 7 and 14 days pretreatment on glucose yield (mg g^{-1}) of *A. donax* biomass by *T. koningii* and *A. niger* at 24, 48 and 72 h of enzymatic hydrolysis. Untreated biomass was taken as control. **(b)** Effect of untreated, sonication 15 min (S15), sonication 30 min (S30), sonication 45 min (S45) and sonication 60 min (S60) of *A. donax* biomass at 24, 48 and 72 h of enzymatic hydrolysis. Untreated biomass was taken as control

Table 2: Estimating effect of untreated, *T. koningii* and *A. niger* pretreated *A. donax* biomass on glucose yield (mg g^{-1}) during enzymatic hydrolysis (h)

Time (h)	Pretreatment	Glucose yield (mg g^{-1})	
		7 days	14 days
24	Untreated	35.7 ± 0.4^{c}	58.3 ± 0.2^{c}
	A. niger	91.7 ± 0.3^{b}	110.7 ± 0.2^{b}
	T. koningii	115.6 ± 0.4^{a}	132.4 ±0.5^{a}
48	Untreated	49.6 ± 0.2^{c}	73.5 ± 0.1^{c}
	A. niger	124.1 ± 0.2^{b}	168.2 ± 0.2^{b}
	T. koningii	159.2 ± 0.5^{a}	192.3 ± 0.5^{a}
72	Untreated	65.9 ± 0.0^{c}	106.6 ± 0.3^{c}
	A. niger	168.6 ± 0.2^{b}	214.8 ± 0.8^{b}
	T. koningii	204.1 ± 0.2^{a}	237.7 ± 0.7^{a}

Results are presented as means of three repeats with the values of standard error (SE). Values followed by different letter within each column are significantly different ($P < 0.05$) by Univariate Analysis of Variance "UNIANOVA" following Tukey's test analysis using Statistical Product and Service Solutions (SPSS) version 23

Table 3: Estimating effect of untreated and sonication pretreated *A. donax* biomass on glucose yield (mg g^{-1}) during enzymatic hydrolysis (h)

Time (h)	Treatment	Glucose yield (mg g^{-1})
24	Untreated	24.6±0.4
	S15	47.9±0.1
	S30	80.7±0.3
	S45	92.3±0.3
	S60	119.9±0.6
48	Untreated	44.5±0.2
	S15	63.1±0.0
	S30	112.5±0.5
	S45	125.4±0.6
	S60	164.9±0.5
72	Untreated	67.1±0.4
	S15	76.1±0.2
	S30	152.6±0.4
	S45	161.4±0.2
	S60	204.5±0.4

*Pretreatments: Untreated, S15; Sonication 15 min, S30; Sonication 30 min, S45; Sonication 45 min, S60; Sonication 60 min

mechanisms of the fuel (Vassilev *et al.* 2010). In current study, fixed carbon content was 20.8% slightly different from previously reported values. It is the valuable characteristic of the biomass as it represents the potential of biomass to release sugars and be used as biofuel source (García *et al.* 2012; Vanja *et al.* 2017). As given in Table 1, the elemental analysis indicated carbon, hydrogen, nitrogen, sulfur and oxygen were found with insignificant difference (Licursi *et al.* 2015; Saikia *et al.* 2015). Upper surface of biomass generally contains hemicellulose, lignin and ash enclosing interior cellulose fiber (Wang *et al.* 2016). Fuel efficacy of biomass depends on the atomic ratio of H/C and O/C. Lower ratio (0.13) found in our results reveals the higher energy content (Singh 2019). Current study shows that *A. donax* biomass comprises cellulose, hemicelluloses and lignin (Table 1), which is sufficient holocellulose to be converted into monomeric sugars and ultimately to bioethanol (Saikia *et al.* 2015; Lemões *et al.* 2018).

It was found that both fungal strains *T. koningii* and *A. niger* produced maximum sugars after 14 days of pretreatment. *T. viride* resulted in conversion of cellulose to glucose as 56% of the theoretical yield after enzymatic saccharification of rice straw pretreated biomass (Ghorbani *et al.* 2015). However, reducing sugar yield obtained by fungal-pretreated cotton stalk (10.91–55.6 mg g^{-1}) reported by Wang *et al.* (2016) was still lower than the current study. Pretreatment of rice straw by *Pleurotus florida* showed total reducing sugars at 353 mg g^{-1} of dry biomass with 75% efficacy at 72 h of saccharification (Naresh Kumar *et al.* 2018). The lignocellulosic waste sawdust at 72 h of saccharification with cellulase enzyme (derived from *T. estonicum)* produced 78.56% glucose (Saravanakumar and Kathiresan 2014).

The enzymatic digestibility following fungal pretreatments, was reported higher as a result of significant lignin degradation (Wan and Li 2012). Sonication pretreatment of *Arundo* biomass for 60 min was found more efficient in producing reducing sugar yield. This is in accordance with the findings reported for maximum cellulose release at 60 min from *A. donax* biomass (Mishra *et al.* 2014). Alkali assisted microwave pretreatment by using cotton stalk biomass, collected 0.495 g g^{-1} reducing

sugars (Vani *et al.* 2012). The higher levels of sugar yield can be attributed to lower lignin portion (Wang *et al.* 2016). The SEM images of pretreated biomass illustrated disintegration and abrasions as compared to untreated biomass. The disruption of the integrated structure after various pretreatments, increased the accessibility of cellulose, which may enhance the effective absorption of enzyme in the interior of cellulose, as is obvious too by the increase in more glucose release from cellulose (Fig. 2). *T. koningii* pretreated biomass was highly susceptible to enzyme degradation as compared to other pretreated biomass and hence, produced highest glucose yield among all the pretreatments. The destruction and disintegration after different pretreatment approaches enhance the accessibility of cellulose, which in return increase efficient absorption of enzyme resulting in maximum fermentable sugars. The SEM images of 60 min sonication pretreatment, exhibited greater disruption of *Arundo* biomass, which also validated our findings of higher glucose yield. These outcomes evidently implied advantage of *T. koningii* pretreatment over *A. niger* and sonication for effective conversion of *A. donax* to bioethanol.

Conclusion

A. donax is rich in cellulose but difficult to degrade into fermentable sugars. In untreated *Arundo* biomass, 72 h hydrolysis glucose yield was found significantly lower than pretreated biomass. SEM observations evidenced advantageous intensive structural changes at varying degrees after pretreatments of *Arundo* biomass. *T. koningii* pretreated *Arundo* biomass had maximum surface disintegration and degradation of fibers, which led to higher fermentable sugar yield as compared to *A. niger*. Enzymatic hydrolysis showed that *Arundo* biomass incubated with *T. koningii* also yielded higher glucose content. *T. koningii* pretreatment proved to be a feasible and eco-friendly alternative for higher amount of fermentable sugars and second-generation ethanol. Sonication is not viable alternate for *Arundo* biomass pretreatment. Prolonged pretreatment is a main barrier for application of fungal pretreatments. Results further showed the effectiveness of fungal pretreatment for improved glucose yield from *Arundo* biomass. Further research studies based on combined pretreatments, characterization of new enzymes, fungal consortiums, cost effectiveness, environmental sustainability, genetic engineering, enzymatic hydrolysis optimization, delignification and enhancement of fermentation are needed to explore more efficient strategies to obtain highest yield of fermentable sugars from lignocellulosic biomass.

Acknowledgments

First author (MS) acknowledges the financial support from Higher Education Commission Pakistan; under "International Research Support Initiative Program" (Project no: 1-8/HEC/HRD/2017/8371). MS also acknowledges COMSATS University, Islamabad, Pakistan for providing scientific facilities; Director Zheng Peng for laboratory and equipment's facilitations at Joint Laboratory of Advanced Applied Technology of Water Treatment, Gansu Academy of Membrane Science and Technology Lanzhou; Abdul Wahab, Habib Ullah, Falak Shair and Zafar Hayat; PhD scholars of Applied Microbiology and Biotechnology Laboratory COMSATS University Islamabad for assistance in handling analytical data and preparing illustrations.

Author Contributions

MU conceived of the presented idea and supervised the research work. SM designed the study, conducted experiments, collected data, performed analytical methods, drafted manuscript. ESS, XKL, FYH and MU supervised the research work. XKL and ESS worked on almost all technical details of this research study and improved write-up. KM and JJ performed analytical computations and took lead for structural analysis. All authors discussed the results provided critical feedback and helped final shape the research, analysis and manuscript.

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