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a bottom-up method towards reducing the quantity of lignocellulosic wastes from environments for valuable products.

Public interest statement

Despite vast reports on the application of different lignocellulose biomass for value-added products, only about 2.5 % from the over 12, 000 tonnes generated annually, from lemongrass, are being used as potential plant for value-added products. Various studies have also recorded success on the application of lemongrass to different fields. Unfortunately, they confined majorly on its applications in agriculture, pharmaceutical, food and flavour, and cosmetic industries. With limited studies on the application of the lemongrass for reducing sugar production, and without the involvement of sodium bisulfite during the physicochemical pretreatment process. This study offers a fabulous method of utilizing the lemongrass leaves by physicochemical pretreatment and enzymatic saccharification for reducing sugar production.

1.0 Introduction

Lemongrass had been classified under kingdom: Plantae (plants), Subkingdom: Tracheobionta (Vascular plants), Superdivision: Spermatophyta (seed plants), Division: Magnoliophyta (flowering plants), Class: Liliopsida (monocotydons), Order: Cyperales/Poales, Family: Poeceae (grass family), Genus: *cymbopogon* and with about 120 identified species, which include *Cymbopogon refractus*, *Cymbopogon citrates*, *Cymbopogon flexuosus, Cymbopogon distans*, *Cymbopogon martini, Cymbopogon nardus, Cymbopogon jwarancusa, Cymbopogon schoenanthus and Cymbopogon tortilis* (Lee et al., 2016, Goëau et al., 2016). Lemongrass is characterized as an aromatic perennial plant with almost six feet (6ft) stem height. The stem is stout, downy, and smooth, stiff, and rigid, and cylindrical in appearance. But the leaves are elongated with the others being reduced to a sheath like structure (Hanaa et al., 2012). Humid and warm climatic conditions coupled with enough sunshine are the most favourable environments for lemongrass cultivation, hence they are predominant to Australia, South-East Asia, South Asia and other African regions (Hanaa et al., 2012, Tajidin et al., 2012). It had been reported to have diverse applications in agriculture, pharmaceutical, food and flavour, and cosmetic industries (Batista, 2014). It has equally been reported to be used as a medicinal plant to remedy nervous and gastrointestinal disorders, so also as analgesic, diuretic, anti-inflammatory, sedative, antispasmodic, anti-pyretic (Santin et al., 2009, Hassan, 2016, Batista, 2014), as anti-fungal and anti-microbial activities, antidepressant and

antioxidant (Matasyoh et al., 2011, Batista, 2014). It is also consumed as tea to mitigate gut and stomach disorders as well as mood enhancer (Hanaa et al., 2012, Hassan, 2016). Moreover, research has described lemongrass leaves with high potential to essential oils production. The essential oils extracted out of lemongrass are natural volatile liquid with aromatic flavour which derived attention in perfume and flavouring factories (Tajidin et al., 2012, Boukhatem et al., 2014, Batista, 2014).

Lemongrass leaves is considered among the lignocellulosic plants and is reported to constitute mostly of lignin (11%), Volatile oil (0.2-0.4%), myrcene (12-20%), hemicellulose (28.5%), ash (11%), cellulose (29.9%) and crude protein (5.1%) by dry weight (dos Santos Barbosa et al., 2008) out of which the lignin, cellulose and the hemicellulose are the foremost concern components of the lignocellulose that require more attention (Ang et al., 2015). This could be due to their rigid structures that needed to be altered when a plant, like lemongrass is considered as substrate to produce variable products (Hussin et al., 2015), like reducing sugars. Reducing sugars have been discovered among the abundant constituents of various lignocellulosic materials. It was found to be embedded within cellulose from the lignin-hemicellulose matrix of the plant materials, which requires certain level of pretreatments before it could be extracted out from the carbohydrate matrices (Ravindran and Jaiswal, 2016, Kumar et al., 2009). Based on the vast reports on the application of lemongrass leaves to various fields, only about 2.5 % from the total generated annually (over 12, 000 tonnes) are being used as potential plant for value-added product. Majority from the total harvest annually are either burnt or disposed on the farmlands, which would eventually cause some environmental hitches. Despite enormous literature that have been available on the presence of compounds like reducing sugars from various agro-based residues, the use of lemongrass leaves for total reducing sugars production has not been excessively investigated. Therefore, this research has been focused to evaluate the potentials of lemongrass leaves for value-added products like total reducing sugar through the optimization processes of the lemongrass leaves (LGL) using physicochemical and enzymatic saccharification.

2.0 Materials and Methods

2.1 Collection and Preparation of Lemongrass Leaves

The LGL were obtained in farm at Taman Universiti, Johore Malaysia. They were put in bags, washed with distilled water to remove other debris. The LGL were sliced into small fragments and oven-dried for 6-10 days at 60 °C. They were then made to powder and sifted to obtain smaller units that passed through125 µm mesh and kept in sealed plastic containers at 20 °C with 10 % moisture content wet basis (w.b) for further use (Tumuluru et al., 2015).

2.2 Lemongrass Leaves Physicochemical pretreatment

Production of reducing sugar using the liquid hot water with sodium bisulfite (physicochemical) pretreatment was conducted in shake flasks by using the method put forward by Hussin et al. (2015). The process was commenced by pouring 8 % w/v from the mechanically ground lemongrass leaves powder into shake flasks containing distilled water. 0.5 % w/v of sodium bisulfite was added and the contents were mixed thoroughly before putting into water bath at 100 °C for 60 min. They were then allowed to cool before being centrifuged for 30 min at 4,000 rpm, 4 °C for the separation of the supernatants and the pellets. The supernatants were collected in different shake flasks for analysis using DNS, while the pellets were resuspended in sterilized water before being placed to dry in an oven for 4-5 days at 50 °C for analysis of the components.

2.3 Lemongrass Leaves pretreatment using Enzymes

Enzymatic pretreatment for production of reducing sugar was performed in shake flasks based the method described by Jiang *et. al.,* (2018). Initially, 8 % w/v from the dried pellets of the physicochemical pretreatment was dissolved into 0.1 M of sodium acetate buffer in shake flasks and was autoclaved at 121°C for 30 min after adjusting the pH to 5.0 with 1M HCL and 1 M NaOH. The contents were left to cool at room temperature before 1 % v/v of the singles and combined (celluclast and viscozym) were added in an equal ratio (50:50). They were then incubated at 55 °C with shaking speed of 150 rpm for 7 days. The contents were put down to falcon tubes and centrifuged for 30 min at 4, 000 rpm towards separating the pellets and supernatants (Jiang *et. al.,* 2018). The supernatants were analysed for the total reducing sugar concentration from the hydrolysate samples using DNS reagents.

2.4 FESEM Analysis

The assessment of LGL through FESEM was conducted to evaluate the efficiency of the saccharification process on the optimized pretreated against the unpretreated LGL after considering the technique employed by Menon and Rao (2012). Preparation of the sample was conducted by coating the samples with platinum on the coating device for 30 min at 2 kV, 10 mA. Therefore, the results were observed and verified using high resolution (10 kV) for the efficiency of the pretreatment processes (Menon and Rao, 2012, Hussin et al., 2015).

2.5 Determination of Total Reducing Sugar using Dinitro Salicylic (DNS) Reagents

Concentration of the total reducing sugar from the pretreated samples was quantified using Dinitro salicylic (DNS) technique as described by Miller (1959) and adopted with modifications (Ang, 2015). A standard curve with glucose monohydrate was initially prepared for the total reducing sugar determination from the experimental samples.

3.0 Results and Discussion

3.1 Pretreatments for Production of Total Reducing Sugar

Enzymatic pretreatment for total reducing sugar production was performed by saccharification processes. The total reducing sugar (18.34 g/)L was the highest obtained with the combination of the enzymes, celluclast and viscozyme (1:1 % v/v), agitation speed (150 rpm), incubation temperature (55 °C) and incubation time (7 days). It was found higher with 3.3-folds than the concentration obtained with the optimized pretreated LGL using the technique of liquid hot-water with sodium bisulfite as a catalyst, physicochemical pretreatment (5.51 g/L), and 8.4-folds higher than those discovered by direct enzymatic saccharification (2.18 g/L) without prior exposing the substrate to liquid hot water pretreatment technique, as depicted in Figure 1. This shows that the utilization of physicochemical technique during the process of the pretreatment on lemongrass leaves prior to the enzymatic saccharification had tremendously aided in breaking the bridges that existed within the structure of the lemongrass leaves biomass, which facilitates the exposure of the cellulose (Mishra et al., 2018, Zhao et al., 2009, Zhuang et al., 2016), leading to higher production of the total reducing sugar.

The use of chemicals as catalysts was found to improve the cellulose biodegradability through hemicellulose and lignin removal, which reduces the intensity of crystallinity and polymerization of the carbohydrate constituents (Singh et al., 2015, Sindhu et al., 2016). Monlau et al. (2013) have discovered that the use of physicochemical pretreatment technique on lignocellulose materials had contributed to the digestibility of the biomass, which eventually enhanced the accessibility of both hemicellulose and cellulose. Moreover, the utilization of catalyst during physicochemical pretreatment of lignocellulose biomass at pH 5.0 enables the removal of hemicellulose, which improves the biomass enzymatic digestibility with lower formation of inhibitory products like furfural (Morjanoff and Gray, 1987, Zhang et al., 2014). The essence of applying the physicochemical technique during lignocellulose pretreatment is to disrupt the structure of lignin, and thus renders the other polysaccharides (hemicellulose and cellulose) highly susceptible for the next pretreatment (Laser et al., 2002, Lee et al., 2014), particularly enzymatic saccharification.

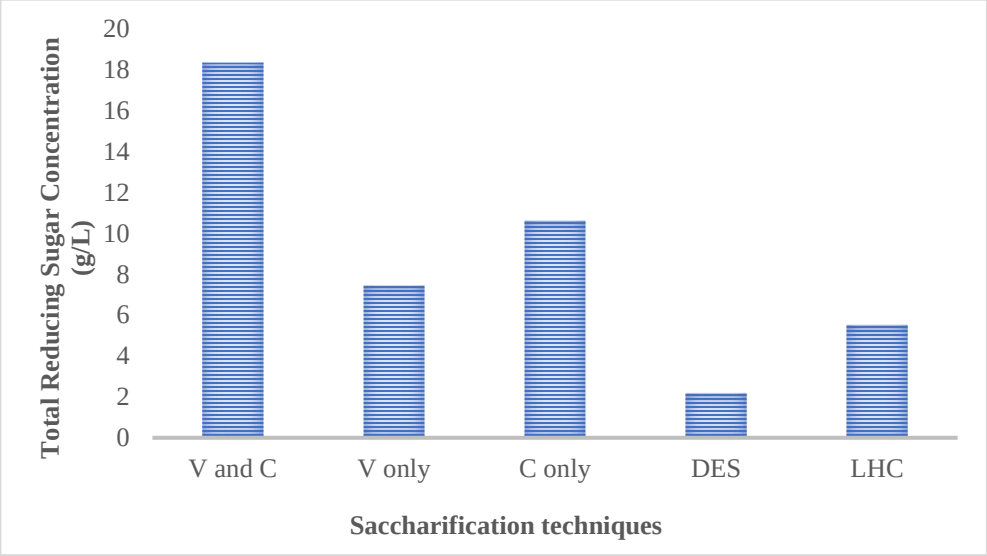


Figure 1 The effect of Various Saccharification Techniques on Lemongrass leaves (LGL) for Reducing sugar Production. Viscozyme (V), Celluclast (C), Direct Enzymatic Saccharification (DES), Liquid Hot water with chemical (LHC). 8 % w/v of lemongrass leave was used for all the experiments with pH 5.0, incubated at 55 °C and shaking speed of 150 rpm for 7 days. Note that V & C, V only, C only, were subjected to physicochemical pretreatment prior to enzymes saccharification. While DES was only used for the enzymatic saccharification after mechanical comminution of the LGL.

Therefore, because only total reducing sugar of 5.51 g/L and 2.18 g/L were obtained with liquid hot water together with the catalyst (LHC) and direct enzymatic saccharification (DES), respectively, when compared to the total reducing sugar obtained using commercial enzyme cocktail (viscozyme and celluclast) (18.34 g/L), viscozyme only (7.43 g/L), and celluclast only (10.62 g/L)(Figure 1). Perhaps due to the complex structure of the lignocellulose biomass, in which the cellulose was shielded by a lignin-hemicellulose matrix that prevents its access easily (Mishra et al., 2018, Singh et al., 2016). Hence the need to augment both the physicochemical and enzymatic saccharification techniques for better performance during the enzymatic saccharification. The process of enzymatic saccharification is well observed among the excellent and attractive techniques towards forming reducing sugar through cellulose degradation. However, the mode of the conversion by the enzyme that catalyses the process is slow, except if the substrates has been exposed to some other methods of pretreatment due to the complex nature of the substrate in which the cellulose was covered by the lignin-hemicellulose matrix (Mishra et al., 2018, Singh et al., 2015).

However, from the application of single enzyme for the saccharification, celluclast was the most efficient which produced 10.62 g/L of the reducing sugar, as opposed to the other single enzyme (Viscozyme) in which 7.43 g/L was obtained. The use of celluclast as a single enzyme for the saccharification on LGL produced the highest reducing sugar as compared to that of using viscozyme as a single enzyme with 1.4-folds, as shown in Figure 3.1. Celluclast was reported with

the attribute of higher affinity to cellulose when compared to both hemicellulose and lignin, which could perhaps be attributed to cellulases content which neutralizes the linkages (β-1,4) that exist on cellulose, and thus facilitates the hydrolysis of the cellulose into fermentable sugars (Singh et al., 2013, Singh et al., 2015, Zhao et al., 2009, Vaithanomsat et al., 2009, Jiang et al., 2018, Ahdno and Jafarizadeh-Malmiri, 2017, Timilsena, 2012). The lower level of reducing sugar obtained with the viscozym as a single enzyme during the saccharification could possibly be due the higher affinity to hemicellulose by which combination of enzymes like hemicellulase, xylanase, β-glucanase and arabinase catalysed the hydrolysis of the hemicellulose into xylose, galactose, arabinose (Wang et al., 2017a, Yang et al., 2008, Yao et al., 2017, Berlowska et al., 2016). This shows that the influence of the hemicellulase from viscozyme was much higher than that of cellulase (Wang et al., 2017b, Berlowska et al., 2016).

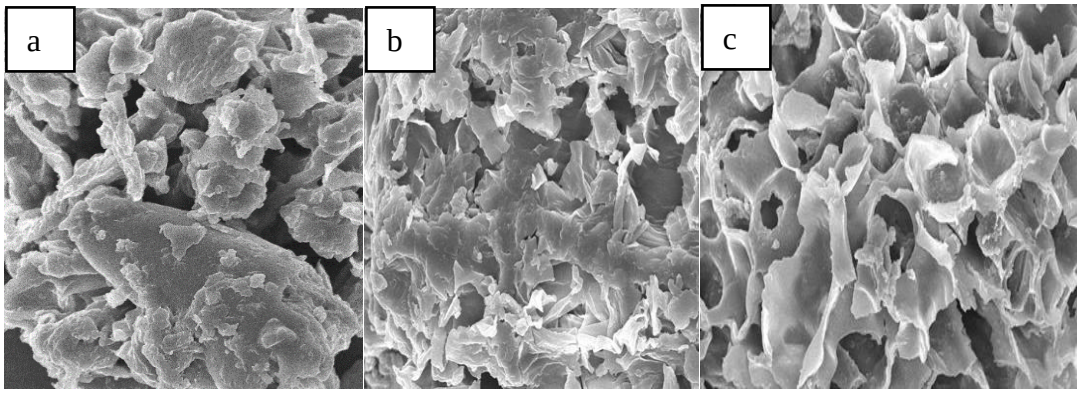
From the evaluation of the efficiency of DES and LHC on production of the reducing sugar, only 2.18 g/L of reducing sugar was obtained from the DES pretreatment technique on the LGL substrate, which was 2.5-folds lower than the LHC method where 5.51 g/L was discovered, as shown in Figure 1. Possibly, the reduction of LGL substrates into smaller particles (125 µm particle size) (mechanical comminution) prior to the enzymatic saccharification did not alter the lignin structure, which is a layer that covers the polysaccharides within the substrate, and thus prevents or restricts their access during the enzymatic saccharification (Wyman, 2018, Jonsson and Martin, 2016). The mechanical comminution process of pretreatment was discovered to primarily takes effect by cutting and grinding the substrate into smaller sub-units, which increases specific surface area, alters the crystallinity of the cellulose, reduces its degree of polymerization, and eventually renders the substrates susceptible to successive enzymatic pretreatment technique (Lee et al., 2014, Sun and Cheng, 2002). However, the process is energy intensive, time consuming and could not efficiently abolish the lignin, which was discovered as the main barrier that limits the accessibility of the other polysaccharides (hemicellulose and cellulose) by cellulases and other pertinent enzymes during enzymatic saccharification (Lee et al., 2014, Zheng et al., 2009). This ensued into lower yield of the hydrolysis. As such, this technique is rarely used exclusively for pretreatment of lignocellulosic biomass without combining with other physical pretreatment technique like liquid hot water, steam explosion etc. (Lee et al., 2014).

3.2 Evaluation of LGL by FESEM

The LGL through FESEM was conducted to appraise the success of the saccharification process on the LGL as revealed in Figure 2. A stiff and smooth fibril of fibres and cells, with elongated strips which restricts the access of the lignocellulose components easily were detected at the surface of the unpretreated (Auxenfans et al., 2017), as shown in Figure 2a. Figure 2a also confirmed a clear change on physical structure on the raw untreated from the direct enzymatic saccharification (DES)

(as seen in Figure 2c) on the LGL. However, because the penetration of the substrates to assess the inner component during the enzymatic saccharification was restricted by lignin, the digestibility of the substrate was only achieved to a certain level (Zheng et al., 2015). This was possibly the reason of lower yield of reducing sugar during the enzymatic saccharification process using the DES, as displayed in Figure1. But changes that have occurred from the morphology of the substrate after the physicochemical technique have indicated a milder disruption of the substrate protective layer, as shown in Figure 2b.

A high degree of morphological and conformational changes on the substrate structure after the enzymatic saccharification was noticed using both enzymes (viscozyme and celluclast), as shown in Figure 2f, when compared to the effects of using these enzymes separately (Figure 2d & e). These interpretations apparently showed that the pretreatment process had tremendously interfered with the lignocellulosic arrangement in LGL structure, thereby unveiling out the cellulose and other lignocelluloses components to facilitate the release of valuable products, including reducing sugar (Bekele et al., 2017, Salleh et al., 2011, Lee et al., 2014). The initial pretreatment of the LGL using liquid hot water with sodium bisulfite had contributed to the cleavage of the glycosidic, ester and ether linkages that exist between the lignin and polysaccharide, which facilitates the combined actions of various enzymes (like xylanase, cellulase, arabinase, hemicellulase and beta-glucanase) present in the enzymes cocktail (viscozyme and celluclast) to enable the release fermentable sugars via further cleavage of the inter- and intra-bonds of the carbohydrates (Mosier et al., 2005, Lee et al., 2014, Wyman, 2018). Therefore, the celluclast used as single enzymes during the saccharification had revealed more conformational changes on the lemongrass substrates, as depicted in Figure 2e. The high affinity of the celluclast to cellulose by cellulases content have contributed to the penetration of cellulose structure through abolition of the linkages that bound the molecules together firmly (Ahdno and Jafarizadeh-Malmiri, 2017, Jiang et al., 2018, Zheng et al., 2015)(Figure 3.2f), thereby increases the release of the reducing sugar to a higher amount when compared to the use of viscozym as single enzyme (Figure 2d).



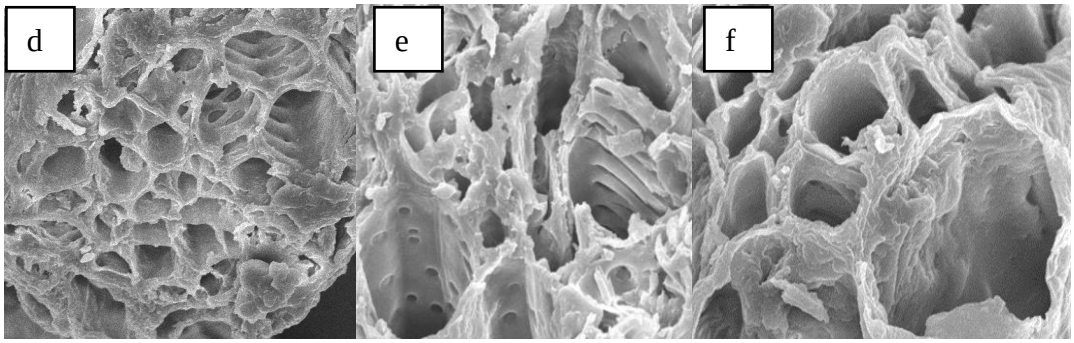


Figure 2 FESEM investigation of the LGL based on the saccharification processes, (1000X): Untreated LGL (a); liquid-hot-water with chemical (b); direct enzymatic saccharification (c); viscozyme only (d); celluclast only (e); viscozyme and celluclast (f).

The use of chemical as catalyst during the liquid hot water pretreatment technique was reported to improve the susceptibility of inner polysaccharides due to the alteration of the structure of lignin from the substrate (Zheng et al., 2009, Zheng et al., 2015, Lee et al., 2014). On the other hand, the use of commercial enzymes on the pretreated LGL (as shown in Figure 2d-f) have formed a disorderly structure ascribed due to exposure of the observed fibres, coupled by the swift wilting of the fibrous lattice, possibly as a results of the biomass cell wall parts solubilisation (Sindhu et al., 2014). The most visible cell wall arrangement would permit better ease of access to the inner carbohydrates by the enzymes during pretreatment by breaching the inner connections amongst the polysaccharides, lignin and other lignocellulose components (Sindhu et al., 2014, Mosier et al., 2005). This might be the main reason behind the higher biomass alterations that were observed in Figure 2 (d, e & f) as compared to Figure 2 (a, b & c) accordingly.

A high degree of morphological and conformational changes on the substrate structure after the enzymatic saccharification was noticed using both enzymes (viscozyme and celluclast), as shown in Figure 2f, when compared to the effects of using these enzymes separately (Figure 2d & e). These showed that the saccharification process had vastly distorts the lignocellulosic arrangement in LGL structure, which unveils out the lignocellulose, carbohydrates and other biomass components to simplify the recovery of valuable products, including reducing sugar (Bekele et al., 2017, Salleh et al., 2011, Lee et al., 2014). The initial pretreatment of the LGL using physicochemical techniques had contributed to the cleavage of glycosidic, ester and ether linkages that exist between the lignin and polysaccharide, which facilitates the combined actions of various enzymes (like xylanase, cellulase, arabinase, hemicellulase and beta-glucanase) present in the enzymes cocktail (viscozyme and celluclast) to enable the release fermentable sugars via further cleavage of the inter- and intra-bonds of the carbohydrates (Mosier et al., 2005, Lee et al., 2014, Wyman, 2018). Therefore, the celluclast used as single enzymes during the saccharification had revealed more conformational changes on the lemongrass substrates, as depicted in Figure 3.2e. The high affinity of the celluclast to cellulose by cellulases content have contributed to the

penetration of cellulose structure through abolition of the linkages that bound the molecules together firmly (Ahdno and Jafarizadeh-Malmiri, 2017, Jiang et al., 2018, Zheng et al., 2015)(Figure 2f), thereby increases the release of the reducing sugar to a higher amount when compared to the use of viscozym as single enzyme (Figure 2d).

4.0 Conclusion

It was determined that the research have provided a bottom-up method towards reducing the amount of lignocellulose residues from the surroundings through production of valuable products. Since the initial pretreatment of the LGL extracts using physicochemical pretreatment have contributed to break of glycosidic, ester and ether linkages that exist amongst the polysaccharide and lignin. This facilitates the combined actions of various enzymes present in the enzymes cocktail (viscozyme and celluclast) to enable the release of the fermentable sugars via further cleavage of the inter- and intra-bonds of the carbohydrates.

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Conflict of Interest Statement

On behalf of all authors, the corresponding authors state that there is no conflict of interest.

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