Research paper

Exploiting ozonolysis-microbe synergy for biomass processing: Application in lignocellulosic biomass pretreatment

Ali R. Mulakhudair [a, b, *], James Hanotu [a], William Zimmerman [a]

[a] Department of Chemical and Biological Engineering, University of Sheffield, Mappin Street, Sheffield, S1 3JD, United Kingdom
[b] The University of Babylon, The Ministry of Higher Education and Scientific Research, Iraq

ARTICLE INFO

ABSTRACT

Pretreating lignocellulosic biomass is an energy and time consuming process. This study presents an alternative pretreatment technique, which explores a synergistic approach between ozonolysis and cellulolytic microorganism-Pseudomonas putida at room temperature. Ozone is a strong oxidative agent that reacts with lignin by attacking the carbon-carbon double bonds, while *P. putida* preferentially hydrolyses the exposed cellulolytic parts of the biomass to simple sugars. The results from SEM and FTIR show a significant reduction in lignin and cellulose contents, leading to relatively high sugar recovery. The glucose concentration increases coincidentally with the ozonation duration and After 24 h however, the concentration reached 1.1 mg/ml, a 323% increase compared with results after 2 h. Increasing the ozonation time to 24 h reduced the biological pretreatment time by 50% but crucially, increases microbial biomass. This approach has potentially high ramifications particularly for industries exploiting lignocellulosic biomass as a feedstock for bioethanol production.

1. Introduction

Lignin present in lignocellulosic biomass is a major barrier to widespread utilization of their carbohydrate content [1]. The lignin represents around 10–30% of the biomass existing in the biosphere, and composed of a set of one or more methyl and hydroxyl groups attached to aromatic rings. The compound is characterised by its amorphous and complex three-dimensional polyphenolic polymer, and fills the interstices between lignocellulosic biomass components, cellulose, hemicellulose and pectin, linking between them in the cell wall of the biomass [2]. Therefore, delignification is vital to enhance enzymatic saccharification and microbial digestion of lignocellulosic residues. Ozonation has been proven as an efficient technique in degrading the lignin polymer, but also helps to oxide carbohydrates concurrently although the rate of reaction with the latter is slower [3]. Ozone is a highly reactive nonlinear triatomic molecule towards compounds containing double bonds and functional groups with high electron densities. Consequently, lignin is most likely to be oxidized in this process as it has high content of

double bonds [4]. The mechanism for carbon-carbon double bond cleavage follows the Criegee mechanism, which predicts the ozonolysis for alkene compounds (C=C) progresses in three different steps [5]. These reactions are fast, and this was proven by observing both the high initial rates of ozone consumption and rapid lignin degradation [1,6]. During ozonation, soluble compounds with low molecular weight mainly organic acids such as formic and acetic acid, are released, resulting in a decrease in the acidification of the solution (~pH 2). The other benefit of the process is that the resulting solution is void of the degradation by-products, which interfere with the downstream processing such as enzymatic hydrolysis with *Pseudomonas putida* and fermentation processing with *Zymomonas mobilis* [7].

However, the efficacy of pre-treatment with ozone depends on the application method. Direct ozone dosing is the most efficient way to deal with such highly reactive and short-lived molecule. Ozone can be dissolved into an aqueous solution from the gas-liquid interface, but this process is mass transfer limited. Traditional methods apply ozone with less attention to mass transfer optimality, resulting in low efficiency and high operating cost. Binder et al. [1], reports that ozonation is one of the most expensive lignocellulosic biomass pretreatment methods. The high operating cost can be reduced however, by exploring an efficient application method. Microbubbles can significantly improve efficiency of

---

dosage due to their high surface area to volume ratio and their low-rise velocity, ensuring maximum gas-liquid contact time. By so doing, substantially cut down on the operating time.

The aim of this work is to increase the biodigestibility of lignocellulosic biomass via ozone-rich microbubble generated by fluidic oscillation. The work also seeks to explore the synergistic performance of ozone and the cellulolytic microbe – *Pseudomonas putida* – in the degradation of lignocellulosic biomass. The study outcome is essential as interest continues on exploring more efficient ways to produce sustainable fuels.

2. Material and methods

2.1. Material and culture medium preparation

Two sets of experiments were conducted: the first was simply pretreatment with ozone-rich microbubble, referred to as microbubble mediated ozonolysis pretreatment (MMO). The second however, was pretreatment with ozone-rich microbubbles followed immediately with microbial application, referred to as microbubble mediated ozonolysis and microbial pretreatment (MMO-M). The untreated wheat straw is referred to as the control.

Wheat straw was mechanically milled to obtain average particle size ~1 mm. The biomass was then washed with distilled water and oven-dried at $80°C$ for 18 h. Wheat straw solution (1% w/v) was prepared with distilled water and the pH set by adding concentrated HCl or 1 M NaOH (Sigma-Aldrich, UK) for the control. The same preparation procedure was observed for the MMO experiment. After MMO pretreatment, the wheat straw was collected and rinsed with distilled water for the MMO-M pretreatment.

The culture medium for the MMO-M experiment was prepared according to Abdul-kadhim and Jarallah [8], with a composition of: 1% MMO pre-treated wheat straw (collected after the MMO experiment), 0.5% yeast extract, 0.02% magnesium sulphate and 0.02% ammonium phosphate dibasic. The medium was then sterilised by autoclaving at $121°C$ and 1 bar for 15 min before cultivating with *Pseudomonas putida* KT2440 at $30°C$ and pH 6 for four (4) days.

2.2. Ozone generation and quantification

Ozone generator (Dryden AQUA, UK) was used to generate ozone and the concentration of the generated ozone was determined using the method described by Rakness et al., [9]. 100 ml/min flow rate was calibrated to ascertain the ozone concentrations and used in all following experiments as it has the highest ozone concentration. Two ozone concentrations – 6.67 mg/L and 8.87 mg/L – were explored at varying exposure times (2, 6, 12, 24 h) to determine a reaction time long enough to allow substantial oxidation of the biomass.

The fluidic oscillator was connected to the ozone generator that fed a sintered glass diffuser (16–20 µm pore size) to produce ozone microbubbles (Fig. 1). Several authors have extensively reported the fluidic oscillator, its mode of operation and application for microbubble generation. Readers are referred to earlier publications of Zimmerman et al. [10], Tesař and Bandulasena [11], Hanotu et al., [12] for more detailed information.

2.3. Analytical methods

Glucose concentration is measured using the protocol described by Miller, [13]. Microbial biomass concentration was determined using the optical density at 600 nm using a spectrophotometer (DTSTM-1700, 1900 NIR) [14].

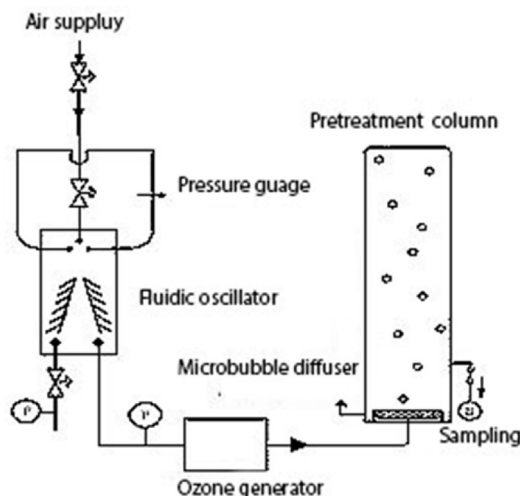Scanning Electron Microscopy (SEM) was used to examine

Fig. 1. Experiment set up. Gas from the fluidic oscillator passes through the fluidic oscillator to the ozone generator. The gas emerges as microbubble-rich ozone.

morphological changes in lignocellulosic biomass. The biomass was oven-dried at $80°C$ for 24 h before coating with gold and imaging at 15 KV with SEM (Model S-360, Phillips).

FTIR-ATR (Perkin Elmer, UK) was used to examine changes in functional groups of the lignocellulosic biomass after pretreatment. These samples were examined at spectrum ranging from 650 to 4000 cm^{-1} and resolution of 4 cm^{-1} with 50 scans per sample. Spectrum Software (V3.3) was used to show the results, background adjusted and normalised at 3300 cm^{-1}.

3. Results and discussions

3.1. Effect of pH and ozone on the functional groups

Fig. 2 presents the FTIR-ATR spectrum for wheat straw biomass after microbubble-mediated ozonolysis (MMO) for 2 h at two pHs (3, 7). Two regions were identified as reaction sites for both ozone and microbial pre-treatment. The first is related to the cellulose content of the biomass, which ranges from ~710 cm^{-1} to ~1100 cm^{-1} wavelength for both amorphous and crystalline cellulose [15–18]. The second region is related to the lignin content of the biomass, ~1595 cm^{-1} [19]. Under acidic condition, the MMO-M pre-treatment (Fig. 2a) resulted in a considerable decrease in both amorphous and crystalline cellulose as well as lignin content, particularly at ozone concentration of around 8 mg/L. There was not much difference however between MMO and MMO-M pretreatment under acidic condition and 6 mg/L ozone concentration (Fig. 2a). Pretreatment under neutral pH (Fig. 2 c and d), only resulted in slight decrease in cellulose and lignin contents of the biomass at ozone concentration around 6 mg/L and at higher ozone concentration, proved counter-productive. Also, there was no observable difference in performance between MMO and MMO-M pretreatments. pH is a system parameter that significantly affects the release and yield of radicals as well as their reaction rate during the ozonation process [20]. Radical species yield increases under acidic condition in comparison with the higher pHs, leading to more effective hydrolysis of organic substrates [20]. Furthermore, microbubble's acceleration of the formation of hydroxyl radicals during ozonation [20], contributes to the improved yield recorded.

The cellulose crystalline ratio (Iα/Iβ), calculated by dividing absorbance at 750 cm^{-1} by absorbance at 710 cm^{-1}, was slightly decreased during all pretreatment combinations, suggesting that

(a)

(b)

(c)

(d)
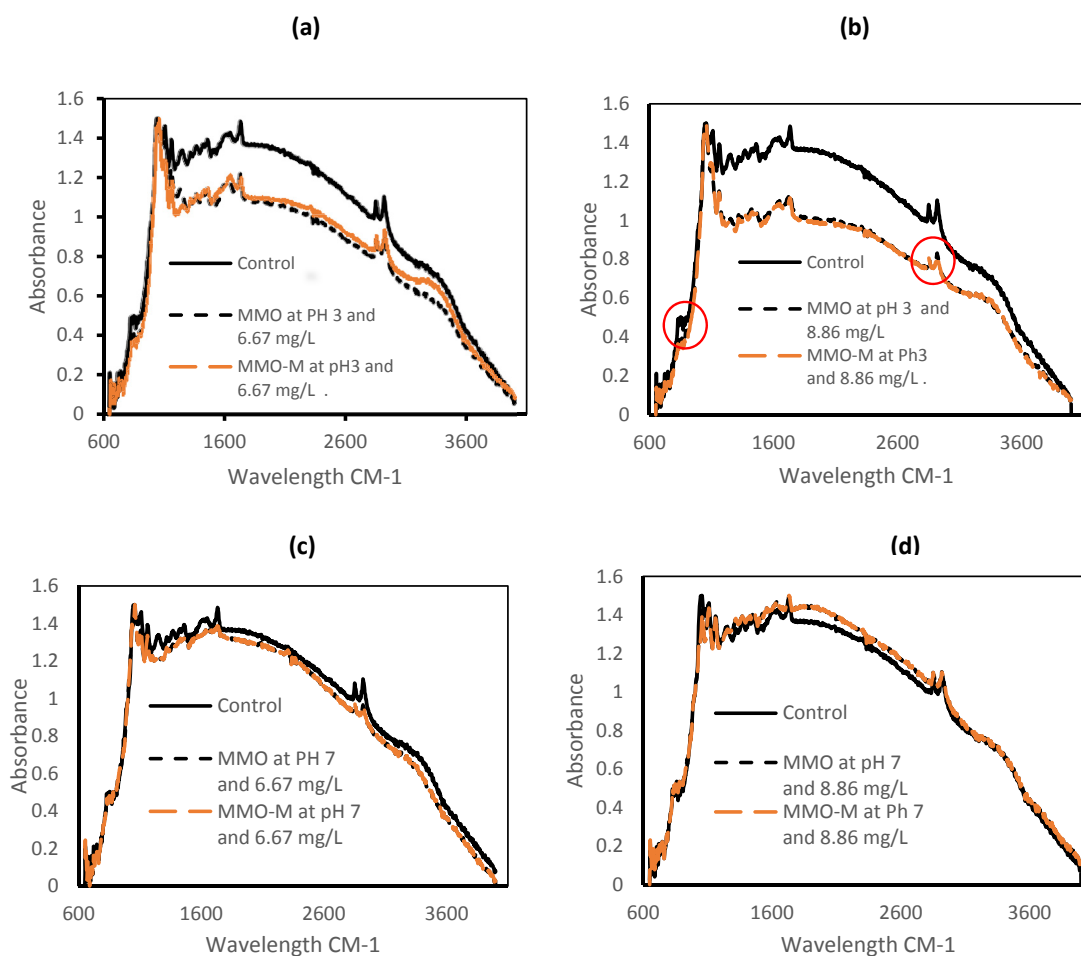
Fig. 2. FTIR-ATP spectrum of lignocellulosic biomass after MMO and MMO-M pretreatments. (a) MMO at pH 3 and 6.67 mg/L ozone concentration and MMO-M at pH 3 and 6.67 mg/L zone concentration (b) MMO at pH 3 and 8.86 mg/L ozone concentration and MMO-M at pH 3 and 8.86 mg/L ozone concentration (c) MMO at pH 7 and 6.67 mg/L ozone concentration and MMO-M at pH 7 and 6.67 mg/L ozone concentration. (d) MMO at pH 7 and 8.86 mg/L ozone concentration and MMO-M at pH 7 and 8.87 mg/L ozone concentration. The readings are representative of triplicate results.

cellulose crystallinity was decreased by ozone. Sakai and Uprichard [21], have reported the effect of ozone on β-glucosidic bonds of cellulose, and showed its cleavage rate as 1.8 times faster than α-glucosidic bonds. This rate relies however, on diffusion rate of ozone in water [21]. The decrease in cellulose crystallinity means decreasing the complexity of cellulose hydrolyses by cellulases. The complexity of crystalline cellulose hydrolyses was reported by Fan et al. [22], as a major barrier to cellulose digestibility by cellulases. Similarly, Park et al. [23], also showed that amorphous cellulose was the easier part of cellulose to hydrolyse.

The crystalline to amorphous cellulose ratio result was obtained by reading absorbance at 1098 cm^{-1} and 900 cm^{-1}. After MMO-M pretreatment, there was a small change recorded in the absorbance at 900 cm^{-1} (circled region in Fig. 2b), which is absorbance of amorphous cellulose. The slight decrease in amorphous cellulose demonstrates the cellulolytic capability of *Pseudomonas putid*a, but crucially, highlights its delignification tendencies. The implication of the latter is that *Pseudomonas putida* can also utilise lignin as a carbon source, and when present with cellulose, causes a diversion in the metabolic activity of the microbe. This outcome corroborates the earlier findings of Mulakhudair et al. [24] who cultured *Pseudomonas putida* in lignin medium and observed an increase in microbial biomass.

Clearly, there was a visible change in absorbance at

1595 cm^{-1}(circled region in Fig. 2b), which is the carbon double bonds absorbance. Carbon double bonds are typically the primary target site for cleavage by ozone. The result agrees with the findings of Kuvshinov et al. [25], and García-cubero et al. [26], who reported that ozone application was effective in attacking carbon double bonds, producing non-toxic esters. Kaneko et al. [27], also reported the selective reaction of ozone with unsaturated carbon double bonds.

3.2. Effect of pH and ozone on the morphological characteristics

Fig. 3 shows a comparison between untreated and treated wheat straw under various pre-treatment conditions. In Fig. 3a, untreated biomass with its physical and morphological integrity are seen to be relatively intact. This is evident in the largely smooth surface with fewer cracks, and densely packed surface with ordered structure.

In contrast, Fig. 3 (b, c, d, e, f, g, h and i) shows morphological changes in wheat straw after MMO pretreatment for 2 h at both pHs (3, 7), and ozone concentrations (6.67 and 8.87 mg/L). From these images, it can be seen that there were substantial changes in the morphology of wheat straw and loss of its structure after pretreatment as well as rugosity on wheat straw surface (Fig. 3 (a, b, c, d and g). However, MMO pretreatment at pH 3 and 8.86 mg/L
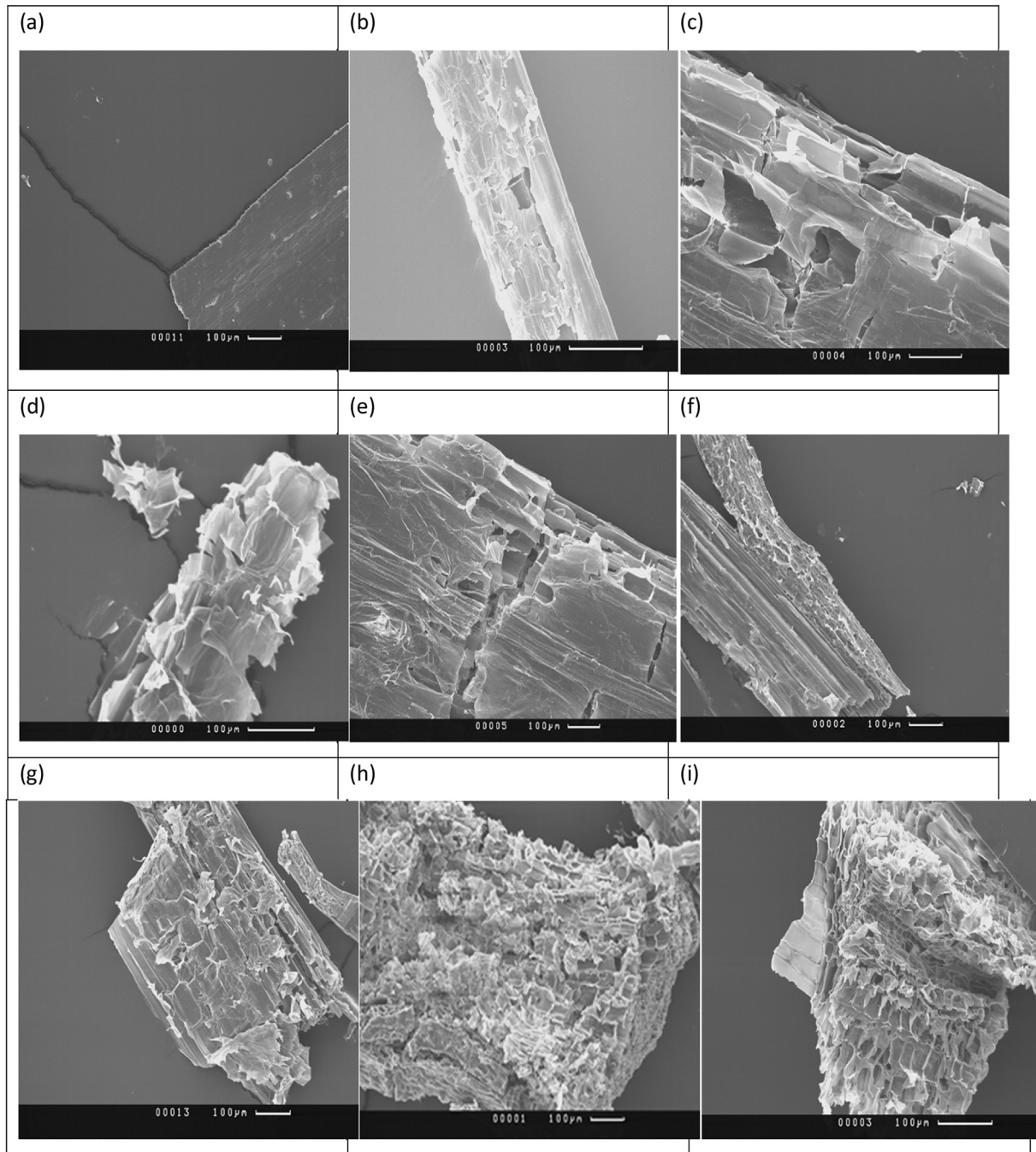
Fig. 3. Morphological changes on wheat straw after MMO and MMO-M pretreatments for 2 h. (a) Untreated wheat straw. (b) MMO at pH 7 and 6.67 mg/L ozone concentration. (c) MMO-M at pH 7 and 6.67 mg/L ozone concentration. (d) MMO at pH 7 and 8.86 mg/L ozone concentration. (e) MMO-M at pH 7 and 8.86 mg/L ozone concentration. (f) MMO at pH 3 and 6.67 mg/L ozone concentration. (g) MMO-M at pH 3 and 6.67 mg/L ozone concentration. (h) MMO at pH 3 and 8.86 mg/L ozone concentration. (i) MMO-M at pH 3 and 8.86 mg/L ozone concentration.

(Fig. 3-h and i) caused a substantial removal of lignin and some of the internal microfibers clearly appeared in comparison with other pre-treatments. Some of these changes have already been previously reported by others authors. For examples, Souza- Correîa et al. [2], observed surface morphology in sugar cane after 4 h of ozone treatment, and confirmed that these changes were due to oxidation by ozone. In addition, De Barros et al. [28] exposed sugar cane bagasse and straw to ozone and observed the formation of multi-porous structures on the biomass surface after treatment for ~1 h. The reaction of ozone with organic substrates is achieved by

ionic cyclo-addition, resulting in the cleavage of unsaturated bonds and activation of the aromatic bonds [21].

Fig. 3i shows morphological changes on wheat straw after MMO-M (at pH3 and at ozone concentration 8.87 mg/L). Holistically, there is a dramatic change in the visual aspect with much debris on its surface. Kristensen et al. [29], described this debris, as part of the middle lamella (composed mainly from hemicellulose) of the cell wall, which separates primary and secondary cell walls in plants. The porous structures observed on the wheat straw used in this study is consistent with the result reported by Mulakhudair

et al. [24], when the authors explored microbubble mediated pre-treatment of lignocellulosic biomass.

Interestingly, glucose was liberated during the MMO process. This is largely attributable to microbubble as the free radicals generated around the gas-liquid interface can attack the lignin, facilitating hydrolysis [24,30]. The glucose concentration measured reached its highest after MMO at 8.87 mg/L and pH3 (Table 1) combined with MMO-M. These observations with other results from FTIR-ATR and SEM strongly suggest that acidic pH and 8.87 mg/L ozone concentration are effective conditions in the pretreatment of lignocellulosic biomass using MMO method.

3.3. Effect of ozone exposure time on lignocellulosic biomass

Results from Table 1 reveals that acidic pH and ozone concentration of 8.87 mg/L gave the best result. Subsequent experiments were conducted under these conditions whilst varying the exposure time (6, 12, 24) h to ascertain the effect of ozone exposure time. It is worth mentioning that in the subsequent tests, the morphological changes on the biomass were not reported, as no difference was noticeable on the physical appearance of the lignocellulosic biomass with prolonged pretreatment beyond 2 h. Therefore, the two parameters monitored in the subsequent experiments, are FTIR-ATR spectrum and glucose yield.

Increasing the MMO pretreatment duration to 6 h was expected to increase the biodigestibility of the biomass, and consequently release more glucose. The results from the FTIR-ATR spectrum (Fig. 4), shows a decrease in both the cellulose and lignin regions respectively. Additionally the glucose concentration produced reached ~0.25 mg/ml at the end 6 h MMO pretreatment (Fig. 5a), five times higher than the glucose produced after 2 h MMO pretreatment (Table 1). With the MMO-M pretreatment, two outcomes were observed during pretreatment: the first is that the microbial

Table 1
Glucose production at varying ozone and pH levels under both MMO and MMO-M pretreatments.

Treatment	Ozonation time (h)	Glucose Produced (mg/ml)±SD	
		MMO	MMO-M
6.67 mg/L at pH7	2	0.0145±0.002	0.071±0.005
8.87 mg/L at pH7	2	0.022±0.007	0.16±0.004
6.67 mg/L at pH3	2	0.015±0.002	0.057±0.008
8.87 mg/L at pH3	2	0.057±0.005	0.26±0.01

Fig. 4. FTIR-ATR spectrum of biomass after MMO and MMO-M pretreatment for 6 h at pH 3 and ozone concentration of 8.87 mg/L. The readings are representative of triplicate results.

density increased, reaching maximum density in <72 h. The second is the decreased glucose level (see Fig. 5b).

Glucose production curve versus time was exponentially fitted to determine the average time constant of action during the MMO. The time constant (τ) of action of ozone on the lignocellulosic biomass to produce glucose was 3.9 h and y0 was 0.188 according to the following equations:

$$y = y0*(1 - \exp\ (-k*t)$$

$$\tau = \frac{1}{k}$$

Where y represents glucose concentration (mg/ml), $y0$ is the initial glucose concentration, t is the treatment time (h), and τ is the time constant.

The reactions kinetics of ozone with an organic compound (lignocellulosic biomass) commonly displays a time constant (exponential constant) during the decay of the reactant (lignocellulosic biomass). This constant characterises the time response of glucose released during the MMO. In other words, time constant gives a timescale for first order kinetics process.

Similarly, further exposure to 12 h MMO pretreatment caused a further decrease in the absorbance for both lignin and cellulose as seen in the absorbance at 710 cm^{-1} to around 1100 cm^{-1} for both amorphous and crystalline celluloses (Fig. 6).

The glucose concentration however, after 12 h MMO pretreatment reached ~0.3 mg/ml (Fig. 7a), greater than five times more glucose produced after 2 h MMO pretreatment (Table 1).

Under MMO-M pretreatment, microbial biomass density increased and reached its maximum in ~72 h. There was an obvious decrease in the glucose concentration. The possible explanation for the result is that liberating glucose in the first pretreatment caused a decrease in the cellulose content of the lignocellulosic biomass, consequently reducing the extractable glucose from the biomass. Additionally, pretreatment of biomass for 12 h with MMO caused a substantial reduction in the main barrier of the biomass, lignin [30], and consequently accelerated *Pseudomonas putida* growth to reach its highest (Fig. 7b).

The change in functional groups after 24 h MMO pretreatment (Fig. 8) shows a considerable decrease in both the cellulose and lignin regions respectively. On the other hand, during MMO-M pretreatment there was a substantial decrease in the cellulose region of the biomass. In addition, the delignification activity by the microbe was observed and can be seen as a reduction in absorbance at 1595 cm^{-1}. *Pseudomonas putida* can concurrently exhibit cellulolytic and delignification activities [24].
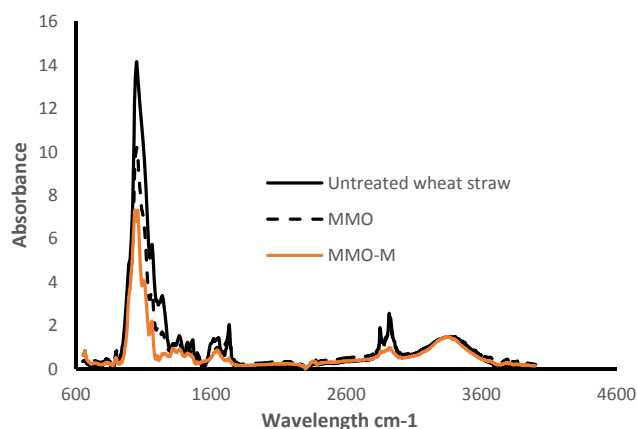
After biological treatment, 85% of the cellulose in straw was degraded. Fig. 9 presents the result of 24 h pretreatment. From Fig. 9a, the glucose concentration reached 0.6 mg/ml, double the concentration at 24 h MMO-M pretreatment. Also, under 24 h MMO-M pretreatment, the *Pseudomonas* strain reached its peak growth density after 48 h (Fig. 9b), a 50% reduction in the pretreatment time in comparison with preliminary result at 2 h ozone pretreated sample (data not shown). Further, this represents ~25% reduction in the pretreatment time in comparison with MMO-M pretreatment at both 6 and 12 h respectively (Figs. 5b and 7b).

Table 2 summarises and presents the comparison between total glucose concentrations after MMO and MMO-M at various treatment durations. There is a clear relationship between glucose yield and pretreatment duration. Increasing the exposure time is proportionate to the glucose production.

The MMO and MMO-M techniques explored in degrading have proved effective in solubilizing lignocellulosic biomass and consequently, releasing glucose crucial for bioethanol production. The
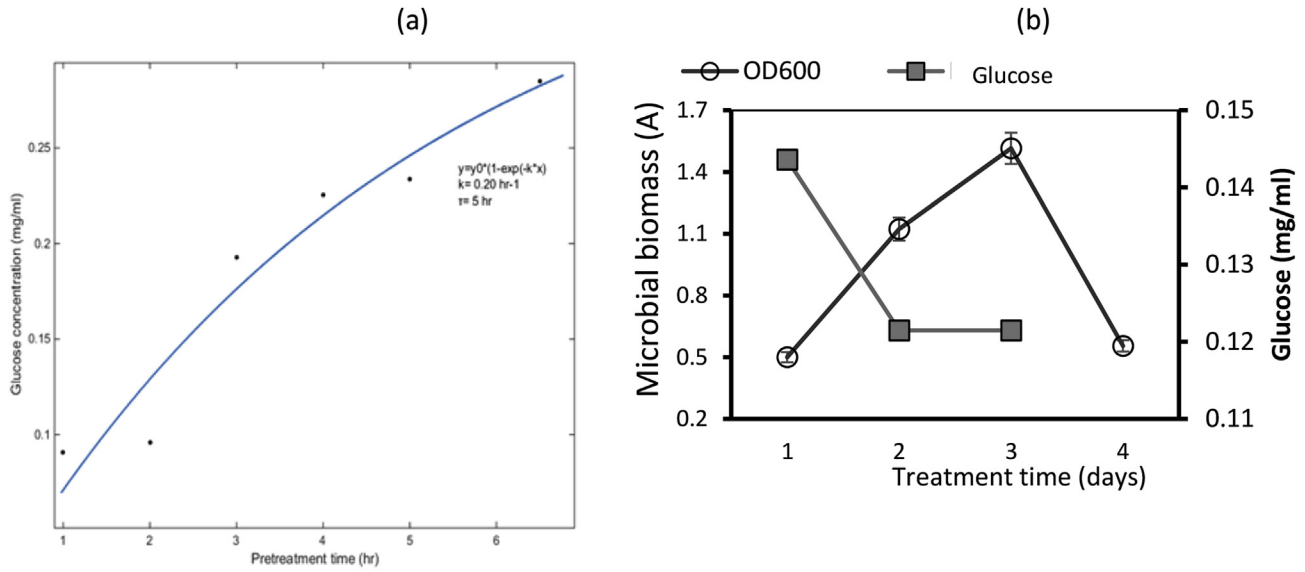
(a)

(b)

Fig. 5. Graph of glucose production after 6 h pretreatment. (a) MMO pretreatment (b) MMO-M pretreatment. Error bars represent standard error for triplicate samples.
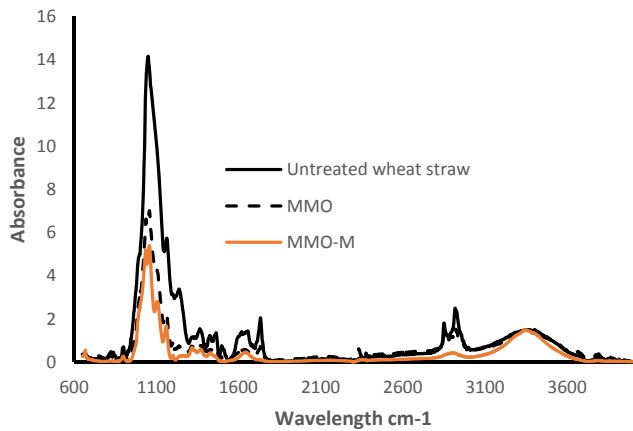

Fig. 6. FTIR-ATR spectrum of lignocellulosic biomass after MMO and MMO-M pretreatment for 12 h at pH 3 and ozone concentration of 8.87 mg/L. The readings are representative of triplicate results.
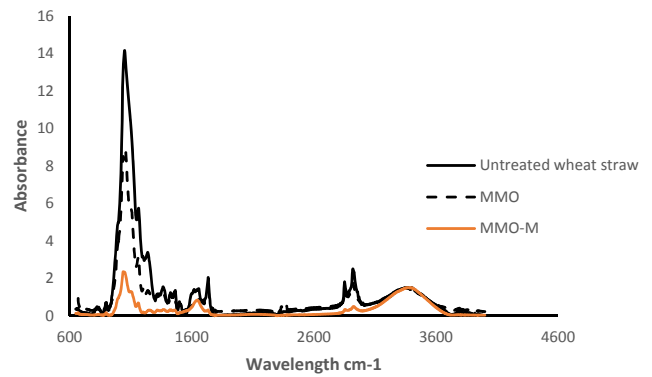

Fig. 8. FTIR-ATR spectrum of lignocellulosic biomass after MMO and MMO-M pretreatment for 24 h at pH 3 and ozone concentration of 8.87 mg/L. The readings are representative of triplicate results.
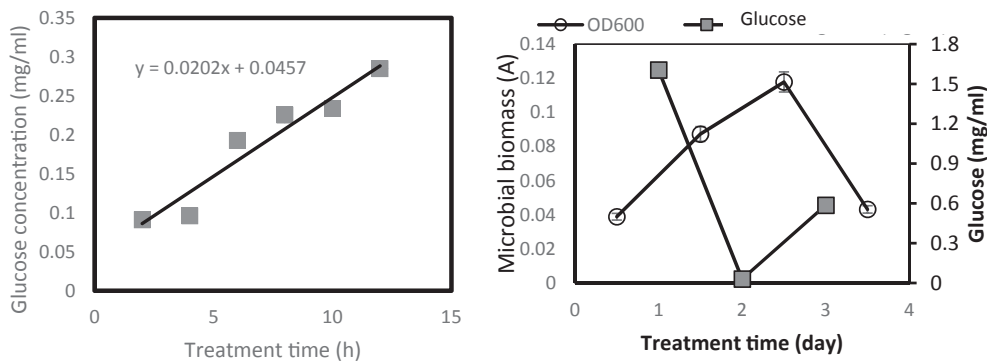

Fig. 7. Graph of glucose production after 12 h pretreatment. (a) MMO pretreatment (b) MMO-M pretreatment. Error bars represent standard error for triplicate samples.

results here are both promising and offer huge prospects on an industrial scale as one of the shortcomings in bioethanol production is not only the high cost of enzymes but also, the difficulty in

effectively degrading lignin with ozone due to the mass transfer limitations. Obviously, some improvements can be made to the study results. For instance, in exploring the limit of ozone
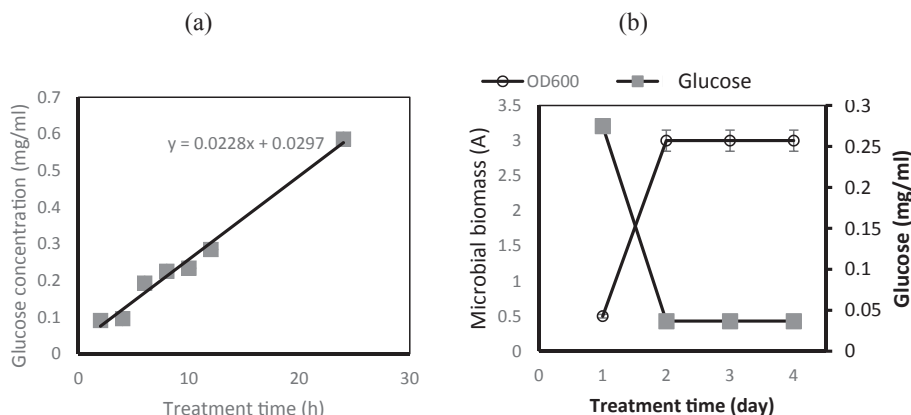
(a)   (b)

Fig. 9. Graph of glucose production after 24 h pretreatment. (a) MMO pretreatment (b) MMO-M pretreatment. Error bars represent standard error for triplicate samples.

Table 2
Glucose production and accumulative produced glucose at the end of MMO and MMO-M of various prolonged exposure times.

Ozonation Time (h)	Glucose Produced (mg/ml)±SD	
	MMO	MMO-M
2	0.057± 0.005	0.26± 0.01
6	0.255± 0.01	0.47± 0.05
12	0.3± 0.06	0.5± 0.07
24	0.6± 0.1	1.1± 0.09

concentration as well as the pretreatment exposure time. Nonetheless, the study outcome provides a significant background for future works.

4. Conclusions

Lignocellulosic biomass from wheat straw was pre-treated with microbubble-rich ozone and a cellulolytic and delignification microbe. Both physical and chemical changes to the biomass were observed. Ozone attacks the carbon double bonds in lignin, substantially degrading it and making cellulose more accessible for hydrolysis. pH, ozone concentration and pretreatment time are all factors affecting hydrolysis and glucose yield with the latter varying directly proportionate with ozone concentration and pretreatment time. Further pretreatment with *Pseudomonas putida* caused considerable changes in both the morphology and functional groups content of wheat straw as well as enhanced the glucose yield.

References

[1] A. Binder, L. Pelloni, A. Fiechter, Delignification of straw with ozone to enhance biodegradability, Eur. J. Appl. Microbiol. Biotecnol. 11 (1980) 1–5.
[2] J.A. Souza-Correîa, M.A. Ridenti, C. Oliveira, S.R. Araújo, J. Amorim, Decomposition of lignin from sugar cane bagasse during ozonation process monitored by optical and mass spectrometries, J. Phys. Chem. B 117 (2013) 3110–3119.
[3] Y. Sun, J. Cheng, Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol. 83 (2002) 1–11.
[4] M.T. García-Cubero, G. González-Benito, I. Indacoechea, M. Coca, S. Bolado, Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw, Bioresour. Technol. 100 (2009) 1608–1613.
[5] R. Criegee, Mechanism of ozonolysis, Anyew. Chem. Inrernal. Ed. 14 (1975) 745–752.
[6] R.A.D. Mbachu, R.S.J. Manley, Degradation of lignin by ozone. I. The kinetics of lignin degradation of ozone, J. Polym. Sci. Polym. Chem. Ed. 19 (1981) 2053–2063.
[7] S.C. Iglesias, Degradation and Biodegradability Enhancement of Nitrobenzene and 2,4-dichlorophenol by Means of Advanced Oxidation Processes Based on Ozone, PhD thesis, Univ. Barcelona, 2002.
[8] A.R. Abdul-kadhim, E.M. Jarallah, Screening of cellulase activity produced from Pseudomonas fluorescens, J. Babylon. Univ. Appl. Sci. 21 (2013) 849–854.
[9] K. Rakness, G. Gordon, B. Langlais, Guideline for measurement of ozone concentration in the process gas from an ozone generator, Int. ozone Assoc. 18 (1996) 209–229.
[10] W.B. Zimmerman, V. Tesar, S. Butler, H.C.H. Bandulasena, Microbubble generation, Recent Pat. Eng. 2 (2008) 1–8.
[11] H. Tesař, V. and Bandalusena, H. Bistable diverter valve in microfluidics, Exp. Fluids. 50 (2.
[12] J. Hanotu, H.C.H. Bandulasena, W.B. Zimmerman, Microflotation performance for algal separation, Biotechnol. Bioeng. 109 (2012) 1663–1673, 011) 1225–1233.
[13] G.L. Miller, Use of Dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–428.
[14] E. Douka, A.I. Koukkou, G. Vartholomatos, S. Frillingos, E.M. Papamichael, C. Drainas, A Zymomonas mobilis mutant with delayed growth on high glucose concentrations, J. Bacteriol. 181 (1999) 4598–4604.
[15] H. Yamamoto, F. Horii, A. Hirai, In situ crystallization of bacterial cellulose II. Influences of different polymeric additives on the formation of celluloses Iα and Iβ at the early stage of incubation, Cellulose 3 (1996) 229–242.
[16] C. Boisset, H. Chanzy, B. Henrissat, Digestion of crystalline cellulose substrates by the Clostridium thermocellum cellulosome: structural and morphological aspects, Biochem. J. (1999) 829–835.
[17] P. Kumar, D.M. Barrett, M.J. Delwiche, P. Stroeve, Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production, Ind. Eng. Chem. Res. 48 (2009) 3713–3729.
[18] L. Laureano-Perez, F. Teymouri, Understanding factors that limit enzymatic hydrolysis of biomass, Appl. Biochem. Biotech. 121–124 (2005) 1081–1100.
[19] I. Bauer, N. Max, 2, 4-dioxygenases catalyzing N-Heterocyclic-Ring cleavage and formation of carbon monoxide, Eur. J. Biochem. 583 (1996) 576–583.
[20] M. Ragnar, T. Eriksson, T. Reitberger, Radical formation in ozone reactions with lignin and carbohydrate model compounds, Holzforschung 53 (1999) 292–298.
[21] K. Sakai, J.U. Uprichard, Ozone degradation of cellulose model compounds, Eur. J. Fat. Agr., Kyushu Univ. 36 (1991) 45–53.
[22] L. Fan, Y. Lee, D. Beardmore, Major Chemical and Physical Features of Cellulosic Materials as Substrates for Enzymatic Hydrolysis, Department of Chemical Engineering, Kansas State University, USA, 1980.
[23] S. Park, J. Baker, M. Himmel, Research cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance, Biotechnol. Biofuels (2010) 3–10.
[24] A.R. Mulakhudair, J. Hanotu, W. Zimmerman, Biomass and Bioenergy Exploiting microbubble-microbe synergy for biomass processing : application in lignocellulosic biomass pretreatment, Biomass Bioenergy 93 (2016) 187–193.

[25] D. Kuvshinov, A. Siswanto, W. Zimmerman, Microbubbles enhanced synthetic phorbol ester degradation by ozonolysis, Waset. Org. (2014) 78–81.

[26] M.T. García-cubero, G. González-benito, I. Indacoechea, M. Coca, S. Bolado, Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw, Bioresour. Technol. 100 (2009) 1608–1613.

[27] H. Kaneko, S. Hosoya, K. Iiyama, J. Nakano, Degradation of lignin with ozone: reactivity of lignin model compounds toward ozone, J. W. Chem. Techno 3 (1983) 399–411.

[28] R.D.R.O. De Barros, R.D.S. Paredes, T. Endo, E.P.D.S. Bon, S.-H. Lee, Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw, Bioresour. Technol. 136 (2013) 288–294.

[29] J.B. Kristensen, L.G. Thygesen, C. Felby, H. Jørgensen, T. Elder, Cell-wall structural changes in wheat straw pretreated for bioethanol production, Biotechnol. Biofuels 1 (2008) 1–5.

[30] U. Doe, Breaking the biological barriers to cellulosic ethanol: a joint research agenda, a research roadmap resulting from the biomass to biofuels workshop sponsored by the U.S, Dep. Energy. Biofuels Jt. Roadmap (2006) 38–56.