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Research paper

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

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Abstract

The potential of lignocellulosic biomass as a sustainable biofuel source is substantial. The development of an efficient and cost effective pretreatment approach remains challenging. In this study, we have explored a new, relatively cheap pretreatment option that works at ambient temperatures. By using microbubbles generated by fluidic oscillation, free radicals around the gas-liquid interface of the microbubble readily attack and degrade lignocellulosic biomass, rendering it more amenable to digestion. The combination of microbubbles and *Pseudomonas putida*—a robust delignification and cellulolytic microbe, further improved biomass degradation and consequently, increased glucose production from wheat straw in comparison to solo pretreatment of the biomass with microbubbles and *Pseudomonas putida* respectively. The microbubble-microbe approach to make biomass more amenable to sugar production is potentially a valuable alternative or complementary pretreatment technique.

1. Introduction

Lignocellulosic biomass is gaining increased industrial application due to its abundance and advantages as a raw material for valuable chemical production [1]. Apart from the cost effectiveness and low environmental impact [2], lignocellulosic biomass also represents a crucial option as a renewable energy alternative. Generally, before these benefits can be fully exploited, the biomass must be processed from its raw form to release sugar, the essential nutrient for microbial metabolism.

Owing to the structural complexity of lignocellulosic biomass, however, pretreatment is ideally the first processing option for sugar extraction [3]. Various pretreatment methods have been reported previously, which are classed into thermal, mechanical, physicochemical, and electrical methods [4]. Others Talebnia et al. [5], and Agbor et al. [6], have grouped these methodologies into: physical, physico-chemical, chemical and biological methods.

Regardless of the group, conventional pretreatment methods are energy intensive given the high temperatures and pressures involved especially the physical and thermochemical processes. For instance, Kumar et al. [3] reported that the dilute-acid hydrolysis process requires temperatures and pressures of up to 230 °C and 10 atm respectively. Conversely, biological pretreatment methods offer the advantage of cost effectiveness but this benefit is readily offset by the substantial time consumption of the process. Many chemical methods are simply inefficient, or suitable for some biomass, with low yield and quality [3]. Another concern with traditional methods is their unsuitability for continuous large-scale production. This is important if lignocellulosic biomass is to play a significant role as a consumer goods raw material. Furthermore, heating vessels and variable water pumps account for majority of the capital and operating costs. There are also high costs due to commercial enzyme utilisation.

The key challenge, therefore, is to change from high power and time-consuming systems to energy efficient; time-saving approaches without diminishing biomass yield or quality. One interesting option is the application of microbubbles. Microbubbles offer increased surface area to volume ratio, and have been exploited for their mass and momentum transfer capability in algal culture [7] and microbial harvest [8] and water treatment [9]. Another unique attribute of microbubbles is their low-rise velocity, facilitating efficient dissolution of their content gas in their immediate environment. More importantly, however, microbubbles are known producers of free radicals in aqueous solution [10] [11] [12]. This

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can aid the decomposition of lignin, thereby increasing the digestibility of lignocellulosic biomass. Li et *al.* [11], reported a ~60% decrease in phenol during a 2 h study using microbubbles. In a different study, the authors observed the degradation of polyvinyl alcohol by collapsing microbubbles. Other works have shown microbubbles to be a strong oxidizing/degradation agent [12].

This study sets out to explore the performance of microbubbles and a cellulotic microbe (*Pseudomonas putida* KT 2440) in the pretreatment of lignocellulosic biomass at room temperature. The cellulolytic activity of *Pseudomonas putida* has been previously reported [13][14][15]; however, its application as the main cellulolytic agent is yet unexplored. *Pseudomonas putida* produces enzymes that break down cellulose with the robustness to cope with significant toxicity levels resulting from the biomass. This new approach holds the potential of reducing/eliminating the high costs associated with conventional pretreatment techniques, if it proves effective in digesting lignocellulosic biomass to release fermentable sugar.

2. Materials and methods

2.1. Biomass and growth media preparation

This study used wheat straw as a lignocellulosic biomass owing to its high cellulose and hemicellulose content, 29–35% and 26–32% respectively, with low lignin percent (16–21%) [16] and availability.

Wheat straw was prepared for pretreatment by mechanically reducing biomass particle size to $\leq$1 mm. The biomass was then washed using distilled water and oven-dried at 80°*C* for 24 h. Wheat straw solution (1% w/v) was prepared with distilled water, and the pH was set at 3, using concentrated HCl (Sigma-Aldrich, UK). The growth medium with a composition of: 1% wheat straw, 0.5% yeast extract, 0.02% magnesium sulphate and 0.02% ammonium phosphate dibasic was prepared according to Abdul-Kadhim and Jarallah, [17]. The medium was sterilised by autoclaving at 121°*C* for 15 min and 1 bar pressure before cultivating with *Pseudomonas putida* KT2440.

2.2. Experimental procedure

The experimentation was divided into four (4) groups to study the effects of each pretreatment. The first group was a control group consisting with biomass in liquid at pH 3. The second group was pretreatment with microbubbles. In this case, wheat straw was treated for 3.30 h at pH 3, by sparging with fluidic oscillator generated microbubbles [8]. The time of pretreatment, 3.30 h, was selected after preliminary studies revealed no additional glucose was produced after 4 h. The third group was the combined pretreatment in which wheat straw was pre-treated with microbubbles for 3.30 h and then, the pre-treatment continued with the application of *Pseudomonas putida* for an additional four (4) hours. Conversely, the fourth group entailed pretreatment with only *Pseudomonas putida*.

After pH adjustment of the wheat straw solution to 3, this solution was introduced into the pretreatment column, which was connected to the fluidic oscillator [18]. Fig. 1 shows the experimental rig, which consists of a fluidic oscillator, a micro-porous diffuser and a pretreatment column. On the other hand, the biological pretreatment was achieved using *Pseudomonas putida* KT2440 in a 500 ml Erlenmeyer flask at 30°*C*. All experiments were conducted at room temperature ($\sim$ 25 °*C*), and untreated biomass was used as a control group to compare with other treated biomass. Samples were assayed daily by centrifuging for 15 min at 13000 $\times$ g and then filtered with a syringe filter unit (Whatman® Anotop® 25 Plus syringe filter, pore size 0.2 μm, Sigma- Aldrich) to measure glucose concentration.

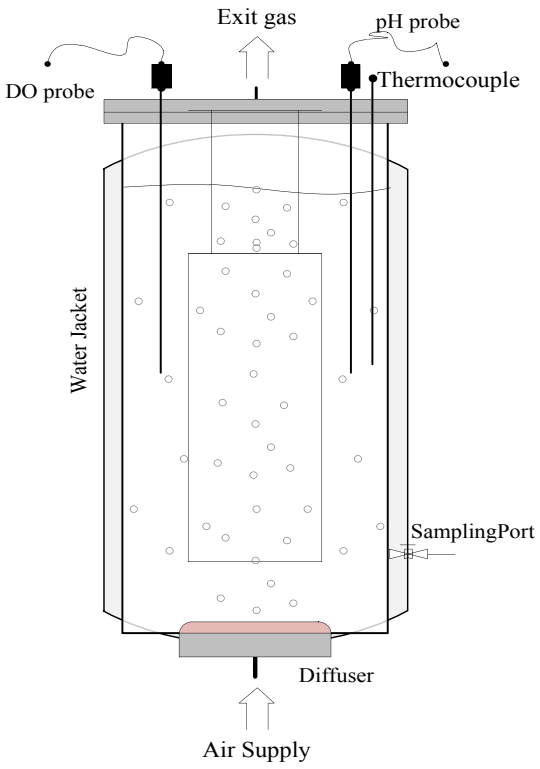

Fig. 1. Schematic representation of the experimental set-up. Compressed air (1 bar) is fed into the oscillator, and there are two outputs from the fluidic oscillator. While, one feeds the microbubbles diffuser, the other is bleed-off.

2.3. Bubble size measurement

The measurement of the microbubbles size distribution was conducted using a Spraytech (Malvern Instrument, UK).

2.4. Glucose concentration

Glucose concentration was measured using a standard glucose assay kit (Sigma-Aldrich, UK) during and after treatment for all experiments.

2.5. Growth pattern of Pseudomonas putida on wheat straw medium

Bacterial growth on wheat straw was monitored optically using a spectrophotometer (DTSTM-1700, 1900 NIR) at 600 nm. The optical density was measured on a daily basis, and the experimental duration was determined based on the measured/observed growth pattern.

2.6. Morphological changes of biomass

Scanning electron microscopy (Model S-360, Phillips) was used to study the morphological changes of the biomass after pretreatment with microbubbles, *Pseudomonas putida* and the combination of both these pretreatment methods. This biomass was coated with gold and set to operate at 15 KV.

2.7. Changes in the functional groups of biomass

FTIR-ATR (Perkin Elmer, UK) was used to examine changes in the functional groups after biomass pretreatment for all experimental

methods. The samples were examined with a spectrum range from 4000 to 650 cm^{-1} and resolution 4 cm^{-1}. The Spectrum software (Version 3.3) was used to show the FTIR-ATR results and to normalise at 3300 cm^{-1}. It is worth noting that after biological pretreatment, the biomass was collected and washed several times with normal saline (0.85%) to remove the bacterial cells, and it was then dried in an oven at 80°C for 24 h before examination with both SEM and FTIR-ATR to avoid their interaction with the readings.

3. Results and discussion

3.1. Pseudomonas putida growth on culture medium

Fig. 2 shows the growth pattern of *Pseudomonas putida* on different cellulosic and lignin media: carboxymethylcellulose (CMC), lignin medium (LM) and more complex biomass, wheat straw medium (WM). Selection of these three media was essential to ascertain the ability of the bacterium in utilising and growing on different carbon sources. While *Pseudomonas* growth on CMC medium can be clearly seen as a monoauxic growth due to the single C-source, growth on LM was a diauxic growth as the two carbon sources (glucose and lignin) are available for metabolism by the microbe. WM culture however, revealed a significant *Pseudomonas putida* growth due to several carbon sources found in wheat straw such as: hexoses, pentoses and their complexes. The higher growth is attributable to the simultaneous utilization of the substrates, which is a typical microbial behaviour when exposed to a mixture of carbon sources [19]. The limitation of carbon and energy sources explains the relative low growth rate and growth pattern recorded for both CMC and LM medium. [20], in contrast to high nutritional performance and typical growth pattern recorded in WM.

It is noteworthy to mention that due to the maximum cellulolytic activities observed after four days, further experimentations on biological pretreatment were conducted for four days.

3.2. Morphological changes on wheat straw

Structural and physical changes are essential indicators of the effectiveness of a pretreatment process. To observe and characterise the morphological changes on the biomass, samples were examined after each pretreatment method under an SEM and result presented in Fig. 3.

Untreated straw (Fig 6 a and b) was observed to be physically intact with its morphology preserved, which is evidenced by the relatively smooth, densely packed surfaces. In contrast, the microbubble (see Fig. 5 for size distribution) mediated treatment (Fig 3c and d) revealed wheat straw with clear porous structures on and through the straw surfaces. The effect results from the decreased rigidity and the re-ordering of fibres during pretreatment. This observation is corroborated by the findings of Cui et al. [21], who reported a similar porous structure on wheat straw after pretreatment with steam explosion at 200–220°C. Microbubble boundaries are highly charged interfaces, carrying and releasing potent free radicals in the containing medium. When in contact with a solid body (particles), the charges are readily deposited and effectively attack the surface, consequently degrading the particle physical structure. Ranger et al. [22], found that generated hydroxyl radicals resulted in abstracting hydrogen atom from the methyl groups or from the carbon in the middle structure of lignin. The extent of surface damage can vary depending on the bubble surface charge magnitude, bubble and particle size and carrier gas. Dosing charge-laden microbubbles can play a crucial role in the catalysis and cleavage of cellulose and hemicellulose and inadvertently, facilitate the release of sugar from the biomass. Glucose concentration measurement reveals a slight increase (0.08 mg/ml after 3:30 h s) with the incorporation of microbubbles during lignocellulosic biomass pretreatment (see Fig. 4a). Typically, long glucose chains in cellulose link with each other via hydrogen bonds and are responsible for the formation of microfiber structures in cellulose. These bonds also connect microfibers with hemicellulose moiety of the biomass [23]. The energy of these interfibrillar hydrogen bonds must be overcome in order to break up these microfibers into separated fibres, and eventually release glucose moieties from the separated fibers [24]. The low result, likely due to the relatively short retention time with microbubbles, demonstrates however, the potential of microbubble in the pretreatment of lignocellulosic biomass.

In Fig. 3 (e and f), there is a significant morphological change on wheat straw after pretreatment with *Pseudomonas putida*. The images reveal the presence of debris – crust-like fragments – covered surface, known as fractions of the middle lamella. The presence of crust-like fragments indicates the absence or partial degradation of hemicellulose since the middle lamella is mainly made from hemicellulose [25]. Improved result (0.159 mg/ml Fig. 3b) here compared to microbubble pretreatment, suggests that enzymes play a more influential role in biomass hydrolyses. Generally, degradation of lignocellulosic biomass by *Pseudomonas putida* is attributable to the secretion of various groups of enzymes. Cellulases attack cellulose chains at random sites to produce glucose as a final product [26]. On the other hand, oxgenases cleave carbon double bonds in lignin by inserting oxygen atoms to form carbon monoxide as a final product [27]. These results are consistent with the findings of Putnina et al. [28], and Cui et al. [21], when the authors pre-treated hemp fibres and wheat straw by steam explosion.

The result of combined pretreatment - microbubbles and *Pseudomonas putida* is presented in Fig. 3 (g and h). Under this pretreatment condition, the wheat straw structure appears relatively loose, largely irregular and highly fibrous. But equally remarkable are the pores observed across the material surface. Similar structural changes were also reported by Gould [29], and Singh et al. [30], who investigated the pretreatment of agricultural residues by alkali delignification combined with microwave. Considering the principal structure of lignocellulosic biomass, lignin is the most complicated component, and poses the greatest physical barrier for biomass hydrolysis. By disrupting the lignin structure of the biomass, microbubbles provide an easier and faster access for the enzymes realised from the microbe for cellulose digestion. The hydroxyl and superoxide radicals generated from collapsed

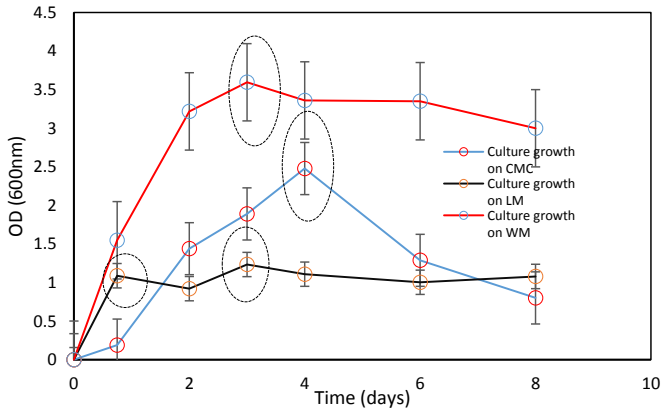

Fig. 2. Plot of growth pattern of *Pseudomonas putida* KT2440 on three different culture media. (a) Carboxymethylcellulose medium (CMC). (b) Lignin medium (LM). (c) Wheat straw medium (WM). Higher growth was recorded with the wheat straw medium with culture peaking in day three. Error bars represent standard error for triplicates.

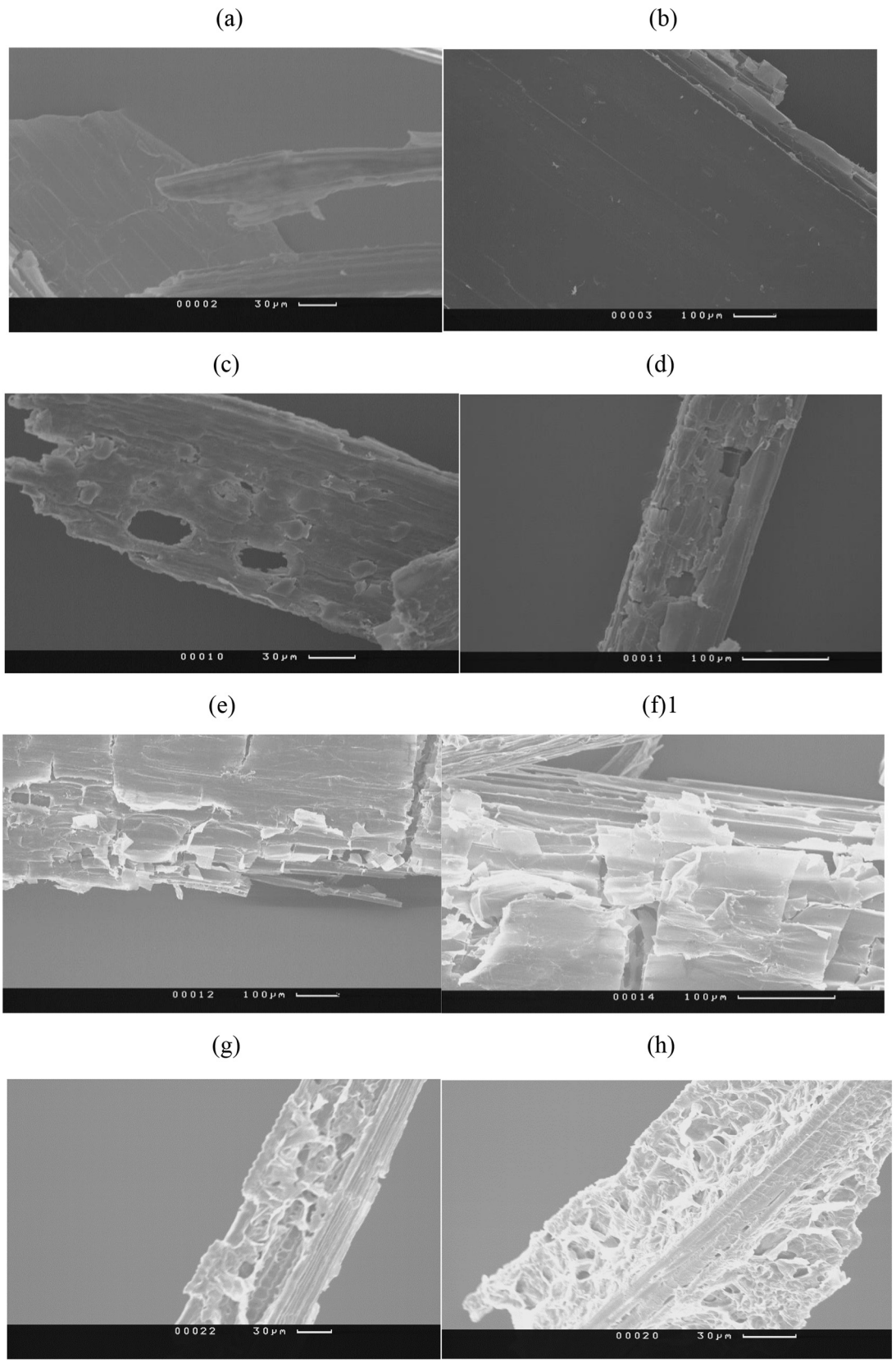

Fig. 3. Morphological changes on wheat straw after different pretreatment conditions. Images (a) and (b) are the untreated wheat straw; (c) and (d) are wheat straw after pretreatment with microbubble; (e) and (f) are the wheat straw after biological pretreatment with *Pseudomonas putida*; (g) and (h) are the wheat straw after the combined pretreatment with both microbubbles and biological organisms.

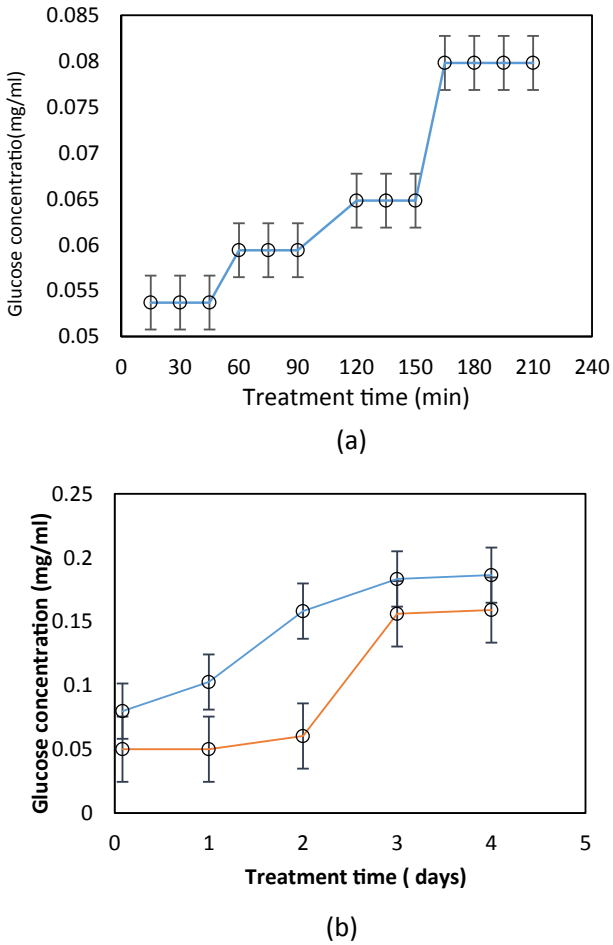

Fig. 4. Plot of glucose concentration with time during pretreatment of lignocellulosic biomass. (a) Microbubble pretreatment. (bi) Biological pretreatment of straw (BTS) (bii) Combined pretreatment of straw (CTS) –microbubble and biological method. Increased glucose yield was recorded for combined pretreatment method. Error bars are representative of triplicate results.

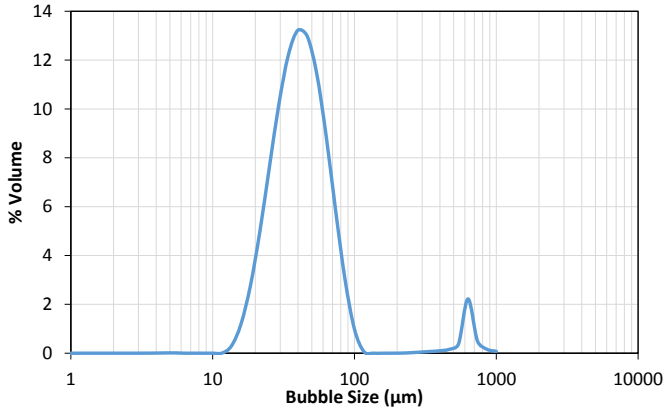

Fig. 5. Microbubble size distribution plot. Sub-100 µm bubbles were largely produced. Second visible peak exists as a result of slight production damage on the microporous sparger.

microbubbles [10] readily attack hydrogen and β-glycosidic bonds in the biomass [11,12]. In addition, hydroxyl radicals are easily transformed into superoxide radicals and vice versa [22], intensifying the metabolic process of *P. putida*.

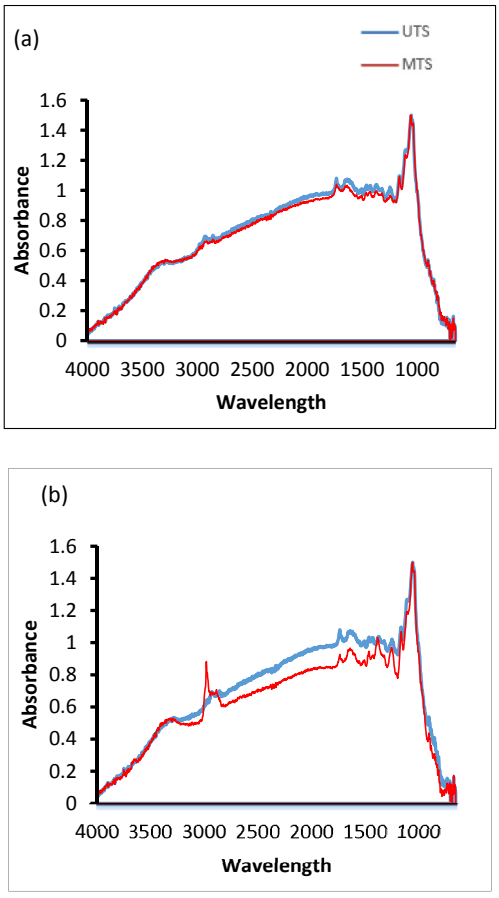

Fig. 6. (a) FTIR-ATR spectrum of biomass after pretreatment with microbubbles (MTS) (b) FTIR-ATR spectrum of biomass after pretreatment with bacterium (BTS). A more pronounced difference in the mid part of the absorbance spectrum, showing the effect of *Pseudomonas putida* on lignocellulosic biomass pretreatment. UTS is untreated samples.

This microbubble-microbe synergy should release more sugar than either of the individual methods. To test this, glucose concentration was measured for the combined pretreatment (see Fig. 4b). Here, wheat straw was first pre-treated with microbubbles for over three hours and the pretreatment continued with the addition of *Pseudomonas putida* for four (4) days. The final glucose concentration obtained from the combined pretreatment, was 0.27 mg/ml. The result of the combined technique is higher than either solo operations. However, the exact order in which the microbubble-microbe delignification of biomass occurs is largely unknown and therefore subject to further investigation.

3.3. Changes in the functional groups of the biomass

Apart from the physical changes, chemical changes to the biomass were assayed to characterise the variations in functional groups under the experimental conditions studied.

Fig. 6 (a) presents FTIR-ATR readings of untreated wheat straw samples and wheat straw after treatment with microbubbles. At 750/710 nm – a ratio of crystalline cellulose (1α/1β) in the biomass [3,31,32] – there was a slight but noticeable decrease in the absorbance, suggesting a crystalline cellulose decrease in the biomass. The decrease was in favour of the microbubble treated samples in comparison with the untreated samples. Boisset et al. [32], observed a similar reduction on cellulosic substrate contents after treating with *Clostridium thermocellum* as did Yamamoto et al.,

[31]. Typically, the crystalline cellulose is more difficult to hydrolyse than amorphous cellulose. Therefore, reducing its amount in the biomass is crucial to the liberation of some inherent glucose molecules and consequently, making the biomass more accessible for further digestion by cellulases [32,33].

Further, the 1098/900 nm ratio defines the ratio of crystalline cellulose to amorphous cellulose. The result reveals a slight decrease in this ratio after microbubbles pretreatment compared with the untreated samples. This decrease was consistent with result at 900 nm, which is related to amorphous cellulose [34]. However, the amorphous cellulose absorbance at 900 nm was unchanged after microbubbles pretreatment. The absorbance at 1745 nm, which relates to the carbonyl groups, the side chains of lignin molecules [35], was slightly decreased after microbubbles pretreatment.

The FTIR-ATR readings for both untreated wheat straw and bacterially treated wheat straw is presented in Fig. 6b. The absorbance of crystalline ratio ($1\alpha/1\beta$) decreased significantly after biological pretreatment compared to the untreated straw. Interestingly, this outcome differs from results from previous studies. For example, Yang et al. [33], reported a decrease in amorphous cellulose degradation by cellulases compared to crystalline cellulose. Fan et al. [36], also reported the inability of cellulases to attack the crystalline portion of cellulose, which led to an increase in crystalline. In their study, the amorphous cellulose content decreased after pretreatment with the bacterium [36] in comparison with untreated samples as well as with different microorganisms [33]. Crystalline cellulose is less hydrolysable than its amorphous counterpart especially to microbes. However, *Pseudomonas putida* is unique for its metabolic versatility, effectively producing enzymes for degrading crystalline cellulose.

Additionally, the current results also revealed a decrease in the absorbance of carbon double bonds content of the biomass at 1595 nm, which is related to the aromatic ring in lignin. Cleavage of the carbon double bond has been reported as one activity of this bacterium. Bauer et al. [27], found that dioxygenase enzymes present in *Pseudomonas putida*, facilitate the cleaving of the carbon double bond in N-heterocyclic rings by inserting oxygen atoms, leading to the formation carbon monoxide as a final product. Also,Tu et al. [37], demonstrated that *Pseudomonas* spp are effective in degrading ciprofloxacin as a result of carbon double bond cleavage via oxidation with manganese.

Further, the 1720 nm absorbance identifies the carboxylic acids or ester groups in both hemicellulose and lignin [38]. The result here shows a decrease in absorbance with the microbe in comparison with the untreated biomass. At 1745 nm, there was a significant reduction in the absorbance in comparison with untreated samples. This absorbance relates to the carbonyl bonds as well as the side chains of lignin [35,39]. Deconstruction of cellulose is problematic by the presence of lignin and hemicellulose as well as their derivatives. Delignification therefore, is an inevitable crucial step in deconstructing cellulose and eventually releasing exploitable monomers for microbial metabolism. *Pseudomonas putida* acts as a delignification agent, effectively decreasing the lignin content and complexes.

Application of microbubbles and *Pseudomonas putida* for the pretreatment of lignocellulosic biomass has improved the hydrolysis of the biomass, showing both physical and chemical changes and the consequent production of glucose.

In order to improve the commercial viability of the pretreatment technique therefore, further work is necessary in understanding the key physicochemical and biochemical mechanisms underpinning the technique and exploring options to improve glucose yield. One option is to increase the surface area of microbubbles by further decreasing bubble size as cellulose is a composite material with surface structure of 3–5 nm size. Dosing with hot microbubble to liberate cellulose is yet another. Crucially however, future studies to investigate methods to improve *P. putida* growth and consequently production of enzyme as the latter play a more influencial role in glucose production is expedient. Provided substantial glucose yield can be obtained, the microbubble-microbe biomass processing technique offers many benefits including: decrease in capital and production cost in comparison to traditional techniques that utilize substantial amount of chemicals and enzymes and high temperature and pressure.

4. Conclusion

An alternative lignocellulosic biomass pretreatment technique using microbubbles and *Pseudomonas putida* at room temperature has been investigated. Physical changes to the biomass structure as well as changes in the functional groups are aspects that were affected by the pretreatment technique. Microbubbles generated free radicals that attacked and disintegrated biomass lignin, making cellulose more accessible for hydrolysis. Further pretreatment with *Pseudomonas putida* caused considerable changes in both the morphology and functional groups content of the biomass, enhancing the glucose yield. The synergy between microbubbles and microbes in biomass processing offers some prospective benefits as a pretreatment technique for biofuel production.

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