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Research paper

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

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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.

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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

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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 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).

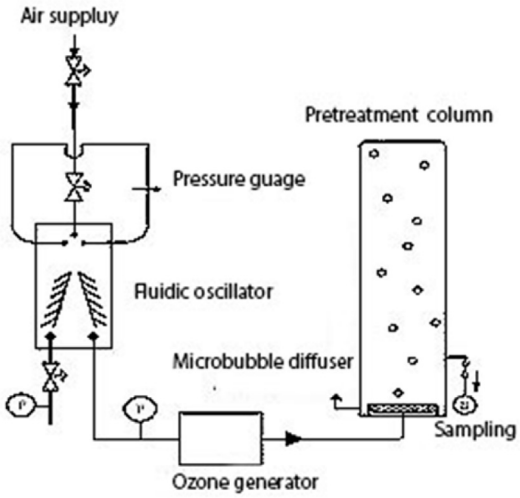

Fig. 1. Experiment set up. Gas from the fluidic oscillator passes through the fluidic oscillator to the ozone generator. The gas emerges as microbubble-rich ozone.

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

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