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Synergism between ultrasonic pretreatment and white rot fungal enzymes on biodegradation of wheat chaff

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ABSTRACT

Lignocellulosic biomass samples (wheat chaff) were pretreated by ultrasound (US) (40 kHz/0.5 W cm^{-2}/10 min and 400 kHz/0.5 W cm^{-2}/10 min applied sequentially) prior to digestion by enzyme extracts obtained from fermentation of the biomass with white rot fungi (*Phanerochaete chrysosporium* or *Trametes* sp.). The accessibility of the cellulosic components in wheat chaff was increased, as demonstrated by the increased concentration of sugars produced by exposure to the ultrasound treatment prior to enzyme addition. Pretreatment with ultrasound increased the concentration of lignin degradation products (guaiacol and syringol) obtained from wheat chaff after enzyme addition. *In vitro* digestibility of wheat chaff was also enhanced by the ultrasonics pretreatment in combination with treatment with enzyme extracts. Degradation was enhanced with the use of a mixture of the enzyme extracts compared to that for a single enzyme extract.

1. Introduction

The need for efficient and clean utilisation of our renewable resources is crucial in the face of population growth, climate change, food security and sustainability concerns. Lignocellulosic residues from the production of wheat and other agricultural crops represent an enormous underutilised energy resource, which can potentially be used as renewable sources of alternative energy to address the increasing demand for energy and concerns over the environmental impact inflicted by the use of fossil-based fuels. These materials which have high carbohydrate content are potential sources of renewable energy as feedstocks for other industries such as feed, organic chemicals, synthetic polymers and cosmetics [1]. Moreover, lignocellulosic biomass does not compete with human food resources constituting an attractive alternative to generate renewable energy and inexpensive compared to the conventional starchy agricultural feedstocks [2]. The removal and effective utilisation of the lignocellulosic residues will not only provide opportunities for agro-industries to reduce their environmental footprint but also enable producers to capture value from an otherwise underutilised waste stream.

The efficient conversion of the energy-rich carbohydrates (cellulose, hemicellulose) in lignocellulosic biomass into accessible sugars is a challenging technically as these materials naturally evolved to resist degradation. This is due to the complex fibrous structures of the materials that have constructed physical barriers to the accessibility of these carbohydrates for enzymatic breakdown. Increasing accessibility to the cellulose/hemicelluose requires degradation of lignin. Pretreatments for degrading the waxy silicate-rich cuticle and lignin sheath, and decrease cellulose crystallinity allow enzymatic hydrolysis of the energy-rich carbohydrates [3,4].

For degradation of lignin, a wide range of thermal, mechanical and chemical pretreatment methods and combinations thereof have been reported [5]. Different pretreatment methods including the use of dilute acid, steam explosion, ammonia fibre explosion, lime and organo solvent pretreatments have been employed to improve enzymatic saccharification [6–8]. However, these pretreatment methods produce undesirable by-products which inhibit downstream processes [7,9,10]. Furthermore, the traditional pretreatments are energy and resource (water) intensive, and cause losses of carbohydrates. Bioconversion with fungal enzymes is safe with low environmental impact. This employs micro-organisms, mainly white and soft rot fungi, actinomycetes, and bacteria which

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degrade lignin under mild conditions [5,11,12]. As biological pretreatment suffers from low efficiency, long residence times, considerable loss of the carbohydrates and high enzyme costs, it would be beneficial if the accessibility of enzymes to the underlying cellulose in lignocellulosic biomass could be enhanced.

The use of ultrasound to promote the pretreatment of lignocellulosic materials has been one such approach. Sonication for pretreatment of lignocellulosic biomass has been shown to enhance the rate of enzymatic hydrolysis and bioethanol production [13–16]. Ultrasonic irradiation of various types of biomass including corn stover [17,18], cassava chip slurry [19], sugarcane bagasse [18], and grain sorghum [20] have shown that ultrasound pretreatment enhances degradation of the biomass by enzymes. In a recent review, Bussemaker and Zhang [21] discussed the application of ultrasound for pretreating lignocellulosic biomass intended for biorefinery and biofuel applications and highlighted the benefits of its application, including lower energy requirements, and increased the accessibility to enzymic hydrolysis and delignification.

The application of ultrasound facilitates mass transfer in addition to the mixing and other effects attributed to cavitation bubbles [14]. The implosion of acoustic cavitation bubbles in water leads to extremely high temperatures and shear forces in the microenvironment. This can bring about significant surface modification in terms of the mechanical damage caused by the asymmetric collapse of the bubbles which cause erosion and abrasion and also the formation of free radicals caused by the breakdown of water on cavitation bubble collapse [22]. The dependence of the physical and sonochemical effects of acoustic cavitation has been observed as a function of ultrasonic frequency. Low ultrasound frequencies (20–100 kHz) favour the physical disruption of the materials while the higher frequencies (>100 kHz) increase free radical generation to sonochemically oxidize components [23].

Although there are several studies using ultrasound, for the pretreatment of lignocellulosic materials, no information focusing on the sequential ultrasound pretreatment approach was reported. Moreover, there have been limited studies of the combined use of ultrasound and lignolytic enzymes to enhance the degradation of lignocellulosic biomasses. One of the most relevant is the work by Yu et al. [24] examining a two-step method consisting of mild ultrasonic treatment step (40 kHz, 250 W) followed by treatment with *Pleurotus ostreatus* for the enzymatic hydrolysis of rice hulls. These authors reported that the combinations led to a significant increase in lignin degradation as compared with that from any single pretreatment, implicating that the efficient breakdown of lignin and cellulose polymers arises from the synergy of enzymatic fungal and physicochemical effects on the biomass. In the biological pretreatment process, fungi (including white-rot), are mostly used to degrade lignin and hemicellulose in lignocellulosic biomass [25].

The present work reports the use of a sequential ultrasound pretreatment in combination with enzyme extracts to enhance the degradation of wheat chaff. Enzyme extracts were obtained from fermentation medium of the wheat chaff by white rot fungi. The application of sequential ultrasound exploits the physical and sonochemical effects of ultrasonication to disrupt the lignocellulosic matrix of the materials. The rationale behind the strategy is the use of low frequency (40 kHz) ultrasound as an initial pretreatment in the sequential process designed to physically disrupt the lignocellulosic matrix of the biomass by cracking and stripping away the waxy layer and teasing the fibrous structure open. The use of 40 kHz as the initial pretreatment frequency in the sequential process was based on the scouring of wool wax, where 35–45 kHz was found to be optimal for cracking the waxy layer and stripping away the scaly surface [26]. From the economic point of view, keeping the sonication time shorter is ideal to minimise the energy consumption. In addition, longer treatment times are known to promote higher lignin condensation on the biomass [27,28]. Generally, an ultrasound pretreatment of 5–10 min has been recognised to be suitable for enhancing the glucose yield in starchy materials, whereas for lignocellulosic material, a sonication time of 10–20 min would be suitable [2]. They suggested that a temperature of 50 °C is appropriate for most enzymes and in addition the effects of cavitation are maximum at this temperature. The effectiveness of the approach was evaluated by measurement of released sugars and phenolic acids from cellulosic and lignin components respectively from wheat chaff, and the *in vitro* digestibility of the treated biomass.

2. Materials and methods

2.1. Materials

Wheat chaff samples were sourced from a local supplier (Country Link, Lara, Victoria, Australia). The samples were then stored at 5 °C prior to the experiments. The wheat chaff samples were used in the experiments as received. The particle size of the wheat chaff pieces were generally of the order of 1–2 cm.

2.2. Ultrasonic pretreatment

The ultrasonic pretreatment was carried out using a Blackstone-Ney Ultrasonics, MultiSONIK-2™ 7 frequency, Ultrasonic Generator (Blackstone-Ney Ultrasonics Inc., Jamestown, New York, USA, 900 cm^2 flat transducer plate, programmable duty cycle, output power and frequency) operating at 40 kHz, 0.5 W cm^{-2}, 50 °C, for 10 min followed immediately by 400 kHz, 0.5 W cm^{-2}, 50 °C for a further 10 min. The operating temperature was maintained within ±3 °C during the experiments, by immersing the transducer in a water bath coupled to a temperature controller (Ratek, Melbourne, Australia) with a water recirculation system. Wheat chaff samples were added into a 250 ml volume conical flask and sonicated in the water bath. The ultrasonic conditions were selected according to previous studies [2,26–28].

2.3. Growth and preparation of enzyme extracts

Trametes sp. strain 2403 and *Phanerochaete chrysosporium* 2403 were obtained from the CSIRO Collection of Wood Decaying Fungi. The isolates were grown in conditions known to optimise their production of lignin-degrading enzymes. Enzyme production media consisted of modified Czapek-Dox broth according to Imanaka et al. [29] (per L: 0.5 g yeast extract, 3 g $NaNO_3$, 0.5 g KCl, 1 g K_2HPO_4, 10 mg $FeSO_4{\cdot}7H_2O$, 0.5 g $MgSO_4$) with 5 mg thiamine-HCl, 5 mg $MnSO_4$ and 0.1% Tween 80 added to optimise conditions for lignolytic enzyme production. Glycerol (2% v/v) was used as a carbon source as it elevates lignolytic enzyme production [30], and prevent production of cellulases in wood-rot fungi [31]. As the current work was specifically focussed on the degradation of lignin rather than cellulose, conditions that promoted lignolytic enzyme production but suppressed cellulase enzymes were preferable. The media was adjusted to pH 4.5 with 10 M HCl.

To obtain larger quantities of the lignolytic enzymes from *Trametes* and *P. chrysosporium* the mycelium obtained from one whole plate of each organism was cut into squares of approximately 2 cm^2 and used to inoculate 1 L of optimised minimal media in a 3 L flask. Three flasks per fungal strain were inoculated. Cultures were incubated at 25 °C and agitated at 150 rpm. After 5 d post-inoculation, the *P. chrysosporium* and *Trametes sp.* cultures were induced with 1.5% sterile wheat chaff or 1.5 mM veratryl alcohol, respectively.