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Research paper

Surfactant-mediated hydrothermal pretreatment of Ryegrass followed by enzymatic saccharification for polyhydroxyalkanoate production

Rashmi Kataria[a,*,1], Trevor Woods[b], William Casey[c], Federico Cerrone[c], Reeta Davis[c], Kevin O'Connor[c], Rohit Ruhal[d], Ramesh Babu[a,b,**]

[a] CRANN and School of physics, Trinity College Dublin, College green, Dublin 2, Ireland
[b] AMBER Centre, Trinity College Dublin, College green, Dublin 2, Ireland
[c] School of Biomolecular & Biomedical Science, University College Dublin, Dublin 4, Ireland
[d] Faculty of Medicine and Health Sciences, University of Antwerp, Belgium

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ABSTRACT

Lignocellulosic biomass from non-food crops is an attractive renewable source of sugars. These sugars can be used as feedstock for the microbial production of different industrially relevant compounds. Delignification of lignocellulosic biomass is a major challenge in sugar extraction. In the present study, ryegrass biomass (non-food), which is high in carbohydrates, was pre-treated with the anionic surfactant sodium dodecyl sulphate (SDS) for effective delignification. Enzymatic hydrolysis of the pre-treated biomass led to the extraction of high sugar levels compared with the raw biomass. Furthermore, these sugars were used for the production of polyhydroxyalkanoates (PHA), which is an alternative to synthetic polymers. Ryegrass treated with 1% SDS solution at 180 °C showed the maximum delignification. This pre-treated biomass was characterised by TGA, FTIR and SEM and showed successful lignin removal. Enzymatic hydrolysis of the resultant pre-treated biomass showed 87% conversion into sugars and was used as a carbon source for the production of medium chain length (mcl) PHAs using two *Pseudomonas* strains. Both strains accumulated approximately 30% of their cell dry weight in mcl-PHAs. Hence, this study shows for the first time that surfactant pretreatment is effective for lignin solubilisation; this process increased the biomass surface area and improved enzyme actions, resulting in higher sugar extraction.

1. Introduction

The global production of lignocellulosic plant biomass, which includes forests, crop residues, grasses, municipal and woods, is 200×10^9 tons per year, though only 8–20 tons per year is utilised (Ragauskas et al., 2006). Lignocellulosic biomass is an organic material derived from a biological origin and composed of cellulose (beta-1,4-glucan fibres), hemicellulose (non-cellulosic polysaccharides such as xylans, mannans and glucans) and lignin (complex poly-phenolic structure). Cellulose and hemicellulose are polymers that can be used as carbon sources for microbial growth. Grass biomass is an attractive feedstock, as it is globally abundant throughout the year and has a low maintenance cost (Kataria et al., 2009). In Ireland, the high abundance of ryegrass allows it to be a promising lignocellulose feedstock for biofuel and biochemical production (Sharma et al., 2012). Ryegrass is the most common bunch type of grass that can survive for several growing seasons and has a 9655.6 kg DM/ha forage production (Oliveira and Gonzalez, 1991). Lignin is one of the most important constituents of this biomass. It is present in the plant cell wall and difficult for microorganisms to use during fermentation, as it is resistant to degradation. The conversion of lignocellulosic biomass to biochemicals is a multi-step process that includes pretreatment, hydrolysis and fermentation. Pretreatment is one of the most important and critical processes in bio-refineries for the production of biofuels and bio-based chemicals (Sun and Che, 2002; Kataria et al., 2013). Pretreatment helps to remove lignin and promote the release of cellulose, which promotes effective hydrolysis by cellulolytic enzymes and makes bio-refinery processes more economical. Several pretreatment methods, including acid, alkali, wet oxidation, ammonia fibre explosion, ozonolysis, microwave-induced hydrothermal, and ultrasonic methods, have been

* Corresponding author at: Dr. Rashmi Kataria, CRANN and School of physics, Trinity College Dublin, College green, Dublin 2, Ireland.
** Corresponding author at: Dr Ramesh Babu P, School of Physics, Trinity College Dublin, College Green, Dublin 2, Ireland. Tel.: +35318962602; fax: +353 1 896 2151.
E-mail addresses: rashmikataria@gmail.com (R. Kataria), BABUP@tcd.ie (R. Babu).
[1] Current addresses: Bioprocess Pilot Facility (BPF), Postbus 198, 2600 AD, Delft, The Netherlands. Department of Biotechnology, Delft University of Technology, 2629 HZ, Delft, The Netherlands.

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employed to destroy lignin (Sun and Che, 2002; Yan et al., 2017; Li et al., 2016). The major challenge with biomass pretreatment is that the decomposition of lignin generates phenolic compounds. Furthermore, the saccharification step, which involves the enzymatic hydrolysis of the pre-treated biomass, can be limited due to the presence of lignin. Lignin absorb a large portion of cellulase and make it unavailable for the cellulosic hydrolysis that results from the extended enzymatic reaction time to achieve a high liberation of sugars (Kumar and Whyman 2009). During pretreatment and enzymatic hydrolysis, surfactants enhance the hydrolysis of lignocellulosic biomass (Eriksson et al., 2002; Lin et al., 2016). When a surfactant is added during pretreatment, it may cause the lignin to be emulsified in the liquid phase and prevent the re-deposition of lignin on the biomass (Qing et al., 2010). Moreover, surfactants have both hydrophobic as well as hydrophilic properties, making them good candidates for lignin removal. Polyhydroxyalkanoates (PHAs) are biodegradable biopolymers with the same properties as conventional synthetic polymers used for plastics. PHAs are intracellularly biosynthesised by different bacterial strains including *Pseudomonas* sp., *Bacillus sp*. and *Methylobacterium sp* (Reddy et al., 2003; Suriyamongkol et al., 2007). The properties of PHA change depending on the monomer composition of the polymer, polymer chain length and length of the side chains. PHAs can be composed of short chain lengths (scl) with 3–5 carbon atoms or medium chain lengths (mcl) with 6–14 carbon atom hydroxyalkanoic acid monomers. The chain length depends on the microbial strain used for synthesis, the carbon source used as the substrate, and the bacterial culture conditions). PHAs are industrially important polymers with various applications including being used in hygiene products, adhesives, films, flavours in foods, dairy products, fabrics and materials and several medical applications (Keshavarz and Roy, 2010). We have already demonstrated that pretreatment and delignification are important for the production of highly digestible biomass and biopolymers (Davis et al., 2013). To the best of our knowledge, few studies have reported on SDS (sodium dodecyl sulphate) pretreatment of ryegrass and its conversion to PHAs. In the present study, SDS, an anionic surfactant, was used for ryegrass pretreatment, and the effectiveness of the pretreatment was determined in terms of the sugars released from the pre-treated biomass by enzymatic hydrolysis. The resulting sugars were evaluated for the production of mcl-PHA using two strains of *Pseudomonas*.

2. Materials and methods

2.1. Materials

Perennial Ryegrass (*Lolium perenne*) was directly harvested from the fields in West Ireland, dried and stored at room temperature. The ryegrass was ground to a particle size of 1 mM, which was utilised throughout this study. The SDS used in the pretreatment experiments was purchased from Sigma-Aldrich (Ireland). Novozyme (Denmark) kindly supplied the Cellic@ CTec2 (cellulase complex), Cellic@ HTec2 (endoxylanase) and NS 22118 (beta-glucosidase) enzymes for the enzymatic hydrolysis of the biomass.

2.2. Biomass composition analysis

Ryegrass biomass composition, including lignin, holo-cellulose, moisture and ash contents, was determined using galvanometric methods. Lignin, moisture and ash contents were estimated according to the methods described in the NEREL protocols (Sluiter et al., 2011). Holocellulose contents (total carbohydrate) were analysed according to a previously described method (Han and Rowell, 1997).

2.3. Surfactant pretreatment

Surfactant pretreatment at different temperatures was performed in a jacket-heated reactor (parr reactor 4843, Parr instruments UK) with controlled temperature and pressure. In the reactor, 5% (w/v) of ryegrass was placed with 1% (v/v) SDS solution at different temperatures (120 °C, 140 °C, 160 °C 180 °C and 200 °C) for 10 min. After the reaction stopped, the solid and liquid residues were separated. The solid residue was washed thoroughly with water and dried overnight at 80 °C until further compositional analysis as well as hydrolysis experiments. Parallel control experiments were also performed with water without the addition of the surfactant at all temperatures. All the experiments were performed in triplicate.

2.4. Sugar estimation

Total reducing sugars (TRS) were estimated using the DNS method (Miller, 1959). One millilitre of sample was mixed with 2 ml of DNS reagent and boiled for 5 min, after which the solution was cooled at room temperature. The optical density (OD) was estimated at 540 nm and the TRS concentration was calculated from a calibration curve (fitted between the glucose concentration and absorbance).

Monomers sugars were determined by HPLC (Dionex ICS-300 system). The column was equipped with a guard column. The mobile phase used was 1 mM KOH at a flow rate of 1 ml/min at 25 °C. The sugar concentrations in the samples were determined from standard sugar calibration curves (glucose, arabinose, xylose, fucose, galactose, and mannose). A suitable dilution of the hydrolysate was prepared and the samples were filtered through syringe filters; then, 10 µL of this filtered sample was used for injecting the samples.

2.5. Biomass characterisation

2.5.1. Thermal characterisation

To determine the decomposition temperature (DT) of the various grass sample fractions, Thermo gravimetric analysis (TGA) was performed on a Perkin Elmer Pyris 1 Thermogravimetric Analyzer. The sample was placed in a platinum pan and heated from 30 to 600 °C at a heating rate of 10 °C/min under nitrogen flow.

2.5.2. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra were recorded on a Fourier transform infrared instrument in the range from 400 to 4000 cm^{-1} for the untreated as well as pre-treated ryegrass fibres.

2.5.3. Scanning electron microscopy (SEM)

The surface morphology of the ryegrass after surfactant pretreatment, water pretreatment and without pretreatment were examined with a scanning electron microscope (Carl Zeiss Ultra model). Different biomass samples were coated with gold before the microscopic observations.

2.5.4. Enzymatic saccharification/hydrolysis of pretreated ryegrass biomass

Hydrolysis experiments were performed in 50 mM sodium citrate buffer with 5% biomass loading and the addition of sodium azide (0.1%) to prevent any microbial growth. To produce the fermentable sugars for PHA production, the biomass was autoclaved at 121 °C for 20 min. The reactions were initiated by the addition of CTec2 [5.4% (w/w of total solid)], HTec2 [0.6% (w/w of total solid)] or NS 22118 [0.5% (w/w of total solid)] (as recommended by Novozyme) to the mixture in the conical flasks. The experiments were performed at 50 °C at 200 rpm in an orbital shaker (Kataria and Ghosh, 2011). The samples were withdrawn at regular intervals and to measure the sugars using the DNS method. All the experiments were performed in triplicate.

2.6. Bacterial culture conditions for PHA accumulation

Two PHA-accumulating strains, *Pseudomonas putida* W619 and *Pseudomonas fluorescens* 555, were used throughout the study. *P. putida* W619 was isolated by Taghavi et al. (2009) and provided by Dr. Jaco