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Enzymatic saccharification of duckweed (*Lemna minor*) biomass without thermophysical pretreatment

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ABSTRACT

Duckweed is a rapidly replicating aquatic plant that has the potential to decontaminate effluent streams from food processing and also has a low-lignin content. Hence it could provide a more suitable source of cellulose for conversion to biofuels.

This paper reports that duckweed biomass has the potential to be enzymatically saccharified to produce glucose and other cell-wall-derived sugars which might be converted to ethanol by fermentation or exploited as industrial platform chemicals. The enzymatic digestibility has been studied on alcohol-extracted, water-insoluble preparations of duckweed cell walls. Within these, glucose accounts for $w = 25.4\%$ (dry wt), which has arisen from cellulose and non-cellulosic glucans including starch. Several commercial cell-wall degrading enzymes and cocktails have been evaluated. Saccharification can be achieved within about 8 h using commercial cellulase at 4.35 FPU g^{-1} substrate in conjunction with added beta-glucosidase at 100 U g^{-1} substrate. The potential for exploiting duckweed is discussed.

1. Introduction

Biofuels are potentially renewable transport fuels which are currently in commercial production. The bulk of biofuel research is focused on the exploitation of (ligno-) cellulosic sources either from non-food crops for fuel, or waste residues from agriculture (second generation biofuels). Lignocellulosic biomass generally contains 55–75% (of DM) carbohydrate [1]. Second generation biofuels are currently uneconomic, largely due to the protective nature of lignin which reduces the accessibility of cellulose by cellulases. Indeed less than 20% of cellulose in native biomass can be enzymatically saccharified unless effective and energy-intensive pretreatments are carried out [2,3].

The duckweeds (family *Lemnaceae*) are the smallest known higher plants. They are very simple, poorly lignified aquatic plants that habit the surface of slow-flowing water for example in ponds and rivers [4,5]. A duckweed plant consists of a small thalloid that floats on the water and which is attached to a simple root structure [6]. *L. minor* appears to obey Kleiber's 3/4-power rule ($G \propto M^{3/4}$) [7] in that they exhibit very much higher growth rates than other larger aquatic plants, with doubling times of between 48 h and 96 h depending on species [5]. They can recover polluting nutrients such as

Abbreviations: AIR, alcohol insoluble residue; BG, beta-glucosidase; CE, Celluclast®; DE, Depol™ 740; WIAIR, water-insoluble alcohol insoluble residue.

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nitrogen and phosphorus from wastewater and have been proposed as providing a sustainable method of wastewater treatments [8,9]. Under appropriate conditions, they can generate biomass more quickly than larger, more traditional, UK crop plants. Culley et al. [10] and Landolt and Kandeler [11] summarize many earlier studies demonstrating the high productivity of duckweeds. Dry weight increases of up to 20 t ha^{-1} y^{-1} are the norm based on results obtained from 25 m^2 lagoons receiving dairy cattle manure [10]. The growing season depends on the location but may enable 10% of the duckweed to be harvested daily in the winter months and up to 35% to be harvested daily in the summer months. Under summer conditions with adequate fertilisation, yields of up to 44 t ha^{-1} y^{-1} have been obtained for cultured *Spirodela polyrhiza* (a large duckweed) in a tank system with correction made for the seasonal effects [12]. These yields compare favourably with those of currently-considered potential energy crops [13], e.g. *Miscanthus* (10 t ha^{-1} y^{-1}), willow (10 t ha^{-1} y^{-1}), poplar (9 t ha^{-1} y^{-1}), switchgrass (12 t ha^{-1} y^{-1}).

The exploitation of duckweed to date has concentrated on the production of gas, oil and biochar through pyrolysis [14] and thermolysis [15]. In addition, researchers in the United States have conducted studies [8] maximising the production of starch in duckweed as an easily hydrolysed source of ethanol as well as a source of protein for use in animal feed [16,17]. However, there seems to be little research into the cell wall composition of duckweed, or on the saccharification of duckweed cell walls for the production of sugars for conversion to either ethanol or other chemicals. Simple compositional analysis indicates that duckweed cell wall material is rich in cellulose and pectin but contains little lignin [18]. Thus, the absence of lignin in duckweed could significantly reduce the pretreatment and enzyme dosages. The cell wall material of duckweed might be an ideal feedstock for digestion with hydrolytic enzymes, a treatment required to release the fermentable sugars that make up the cell wall polymers. The aim of this study has been to evaluate duckweed biomass, as a poorly lignified plant, for its ease of saccharification using commercially-available cell-wall degrading enzymes.

2. Materials and methods

2.1. Plant resource

Duckweed (*Lemna minor*) plants were collected from the surface of the River Yare located close to University of East Anglia, Norfolk, UK (52.61682 N, 1.243815 E) and were sterilised by immersing in diluted sodium hypochlorite (φ = 4%) for 1 min and rinsed with autoclaved distilled water to remove residual bleach. In a sterile containment cabinet, sterilised *L. minor* (10 plants) were transferred into autoclaved Hoagland's E-Medium (100 mL; pH = 5.8) [19] in 250 mL Erlenmeyer flasks stoppered with a sponge.

The inoculated flasks were placed in a growth room under controlled conditions: room temperature (22 °C); five fluorescent light tubes (amount of photosynthetically active radiation: ~120 μmol m^{-2} s^{-1} = 25.7 W m^{-2}) consisting of three conventional tubes (GE F58w 33) and two Sylvania GRO-LUX tubes (F58w/GRO T8) were controlled automatically under long day conditions (16 h light and 8 h dark). The mature plants were harvested at between 6 and 8 weeks after subculturing. No contamination with algae was observed. Fresh plants were used immediately after harvesting, or frozen at −20 °C until required.

2.2. Alcohol insoluble residue (AIR) extraction

Duckweed biomass samples were extracted as alcohol insoluble residues (AIR) in order to remove water, low molecular weight moieties (including mono- and oligo-saccharides, mono- and oligo-peptides, lipid soluble components, low-to-medium chain fats and oils, chlorophyll and some salts). Firstly, fresh (or frozen) plants were ground in ethanol (φ_{final} = 70%) using a pestle and mortar for 10 min to physically break down the cell wall structure. The slurries were transferred to Pyrex® tubes after which they were heated at 80 °C for 15 min. After cooling and recovery by centrifugation (3000× *g*, 10 min), the residue was re-extracted as before in ethanol (φ_{final} = 70%, 80 °C, 15 min) and then once at 80 °C in ethanol (φ = 100%). Finally the AIRs were extracted once in acetone at room temperature and dried at 30 °C overnight. Water-soluble components were removed from the AIR by washing in 0.1 mol L^{-1} sodium acetate buffer (pH 5.0) and then oven dried at between 30 °C and 40 °C to leave water-insoluble AIR (WIAIR) [20].

2.3. Enzymatic hydrolysis

Enzymatic saccharification of the duckweed WIAIR employed three commercial enzyme preparations: Celluclast® (CE; cellulase, Sigma Chemical Co., St. Louis, MO), Novozyme® 188 (BG; Beta-glucosidase, Sigma Chemical Co., St. Louis, MO), and Depol™ 740 (DE; cell wall degrading enzyme cocktail, Biocatalysts Limited, Wales, UK). Depol™ 740 contains mainly ferulic acid esterase along with cellulase and significant xylanase activities. The enzyme activities are defined by the manufacturer for CE and BG as 700 U mL^{-1} [21] and 250 U mL^{-1} [21] respectively, and by Hendrickson et al. (2007) [22] for DE as 170 U mL^{-1}. The FPU activity of cellulase (Celluclast® and Depol™ 740) was also assessed following the standard measurement of cellulase [23]. The enzymes were separately loaded in the designated cocktails, i.e. Depol + Celluclast (DE + CE), Depol + BG (DE + BG), Celluclast + BG (CE + BG) and Depol + Celluclast + BG (DE + CE + BG), in triplicates of each cocktail. Prior to enzyme addition, the CE and DE were desalted using a PD-10 Column (GE Healthcare Life Sciences, Little Chalfont, Bucks., UK) [24] and BG was centrifuged at 16,060× *g* to remove insoluble particulates. Digestions were carried out in triplicate and contained 10 mg of WIAIR and enzyme in 0.1 mol L^{-1} sodium acetate (pH 5.0) containing thimerosal (Sigma Chemical Co., St. Louis, MO; 0.1 kg m^{-3}) in a total volume of 2 mL. The hydrolysis reactions were carried out in Eppendorf vials, and allowed to proceed for up to 24 h at 50 °C with continuous agitation on a Thermoshaker at 120 rpm [25]. Incubations were terminated by heating to 100 °C for 5 min after which the samples were centrifuged at 16,060× *g* for 5 min. The supernatants were recovered by aspiration and frozen prior to analysis. The reducing sugars and glucose were measured subsequently by dinitrosalicylic acid (DNS) and