Production of Deuterated Biomass by Cultivation of *Lemna minor* (duckweed) in D_2O

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Main Conclusion

Common duckweed *Lemna minor* was cultivated in 50% D_2O to produce biomass with 50-60% deuterium incorporation containing cellulose with degree of polymerization close (85%) to that of H_2O-grown controls.

Abstract

The small aquatic plant duckweed, particularly the genus *Lemna*, widely used for toxicity testing, has been proposed as a potential source of biomass for conversion to biofuels as well as a platform for production of pharmaceuticals and specialty chemicals. Ability to produce deuterium-substituted duckweed can potentially extend the range of useful products as well as assist process improvement. Cultivation of these plants under deuterating conditions was previously been reported to require addition of kinetin to induce growth and was hampered by anomalies in cellular morphology and protein metabolism. Here we report the production of biomass with 50-60% deuterium incorporation by long-term photoheterotrophic growth of common duckweed *Lemna minor* in 50% D_2O with 0.5 % glucose. *L. minor* grown in 50% D_2O without addition of kinetin exhibited a lag phase twice that of H_2O-grown controls, before start of log phase growth at 40% of control rates. Compared to continuous white fluorescent light, growth rates increased five-fold for H_2O and two-fold for 50% D_2O when plants were illuminated at higher intensity with a metal halide lamp and a diurnal cycle of 12 h light/12 h dark. Deuterium incorporation was determined by a combination of ^1H and ^2H nuclear magnetic resonance (NMR) to be 40 -60%. The cellulose from the deuterated plants had an average-number degree of polymerization (DP_n) and polydispersity index (PDI) close to that of H_2O-grown controls while Klason lignin content was reduced. The only major gross morphological change noted was root inhibition.

Key Words

***Lemna minor*, duckweed, biomass, deuteration, cellulose, nuclear magnetic resonance**

Introduction

Duckweeds of the genus *Lemna* are small, aquatic flowering plants that are found in fresh water world-wide. Various species, particularly *L. minor* and *L. gibba*, are widely used in toxicology tests due to their easy cultivation and quantification (Moody and Miller 2005; Brain and Solomon 2007). They are utilized as a high-protein feed crop both in the natural environment and in aquaculture and agriculture (Porath et al. 1979; Bergmann et al. 2000). More recently large-scale cultivation to provide cellulosic biomass for conversion to biofuels has been proposed based on their rapid growth rates and low recalcitrance to enzymatic saccharification (Zhao et al. 2012; Zhao et al. 2014). Typically, schemes for duckweed biomass production are combined with their established application in waste water treatment (Bergmann et al. 2000; Körner et al. 2003; Liu et al. 2017). Utilization of *Lemna* duckweed as a platform to produce biopharmaceutical products using recombinant DNA technology was proposed (Gadaska et al. 2003; Stomp 2005) and production of recombinant antibodies has been reported (Cox et al. 2006; Firsov et al. 2018).

Lemna spp. are also notable for their cultivation in D_2O-enriched media to produce deuterium-labelled biomolecules and biomass (Cope et al. 1965; Trewavas 1970) and to investigate heterotrophic growth (Yakir and DeNiro 1990). There is increasing interest in development of deuterium substitution for pharmaceutical applications (Halford 2016), particularly following the FDA approval of the first deuterated drugs (Schmidt 2017; DeWitt and Maryanoff 2018). Deuterium substitution is utilized to manipulate the scattering length density of a material, facilitating molecular structural analysis by neutron scattering and diffraction techniques that are increasingly being applied to understand biological systems (Langan et al. 2012). Partial deuteration of *Lemna* biomass (Cope et al. 1965) as well as production of deuterium-labeled DNA (Trewavas 1970) indicated these duckweed species could potentially be used to produce deuterium-labeled biomolecules. However, the initial investigations encountered complications related to toxicity and metabolic changes following transfer of the duckweed to 50% and higher concentrations of D_2O. Since duckweed species of the genus *Lemna* can grow by vegetative reproduction and have simple roots, it was thought that these small plants would be able to adapt to high D_2O concentrations similar to microalgae. Investigation of *Lemna peruspilla* and *Lemna gibba* for production of deuterated biomass found that duckweed could tolerate 50 - 60% D_2O if grown under photoheterotrophic conditions with glucose as a carbon source in addition to CO_2 in ambient air. D_2O inhibition was synergistic with light intensity under the conditions used and addition of kinetin, a phytohormone that breaks dormancy, was needed to stimulate growth in 60% D_2O. Glucose supplementation improved growth in 50 - 60% D_2O while achieving fixed deuterium incorporation levels in the range of 32 – 56% in the whole biomass with protiated glucose and without addition of kinetin (Cope et al. 1965). Similar to the behavior of terrestrial plants in higher (>30%) concentrations of D_2O, growth was slower and root elongation was greatly inhibited. A subsequent heavy labeling study noted slower growth and shorter roots of *L. minor* plants grown in heavy isotope labeling medium containing 2 mM calcium nitrate-^{15}N, 5 mM potassium nitrate-^{15}N, 10 mM sucrose, and 1 μM kinetin in 50% D_2O under continuous illumination with warm white and daylight fluorescent lamps at an intensity of 1000 ft-c (Trewavas 1970). Despite these inhibitory effects, *Lemna* plants were later reported to adapt to heterotrophic growth in 50% D_2O media over time. Membrane rearrangements of the tonoplast and chloroplast were noted in *Lemna minor* in the first 5 h following transfer to 50% D_2O, but nearly complete recovery ensued after 24 h (Cooke, Grego, et al. 1980). After one week, the cells appeared normal. Protein degradation rates increased and synthesis rates decreased after transfer, but returned to normal levels after 60 h (Cooke, Grego et al. 1979). Protein degradation as a response to the isotopic stress of 50% D_2O resembled that observed for other stressors such as nutrient deprivation (distilled water), nitrate deprivation, and osmotic shock with 0.5M mannitol (Cooke and Davies 1980; Cooke, Oliver, and Davies 1979).

In this study, we demonstrate the production of *Lemna minor* biomass with 50-60% deuterium incorporation containing cellulose with a similar molecular weight distribution (85%) as that found in H_2O-grown controls. These results indicate that this protocol can be employed to prepare highly deuterated plant cellulose and, potentially, other components for experimental investigations and commercial applications.

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Materials and Methods

Cultivation of duckweed Lemna for deuteration experiments

The duckweed strains *Lemna minor* (UTCC490) and *Lemna gibba* (G3) were a generous gift from Biolex, Inc. (North Carolina, USA). The growth medium was Schenk and Hildebrandt's basal salts (Phytotechnology Laboratories, Shawnee Mission, Kansas, USA). House-distilled water was further purified with a Milli-Q system (EMD Millipore, Massachusetts, USA) or with a Barnstead E-Pure system (ThermoFisher Scientific Massachusetts, USA). Deuterium oxide (D_2O, 99.8%) was obtained commercially (Cambridge Isotope Laboratories, Massachusetts, USA).

For preliminary screening, the plants were grown at 23 ° C under continuous illumination with white fluorescent light (Sylvania Daylight F75TBD/B 15W) at 40 μmol m^{-2} s^{-1}.

Carbon source experiments were set up with 50 ml of medium in 250 ml glass conical flasks closed with polyurethane foam stoppers. Each culture was inoculated with 10 fronds. Carbon sources were supplemented at 0.5% v/v. Sodium acetate and sodium succinate stocks were adjusted to pH 4.0 by addition of sodium hydroxide solution. The determination of optimal glucose concentration was carried out similarly, but with 0, 0.5, 1, 2, and 5.0% (0, 27, 55, 110 mM, respectively) glucose in the media.

For perfusion experiments, *L. minor* was grown in an in-house assembled culture system (Fig. 1). The culture system was assembled from 250-ml glass conical flasks fitted with rubber stoppers equipped with inlet and outlet connections. Glass tubing (1/4 inch O. D.) was used to connect the inlet tubing of each flask to a 4-port manifold made of the same tubing via 8-inch lengths of silicon tubing. The manifold was connected with silicone rubber tubing to an air source through an in-line 0.2 micron syringe filter to filter-sterilize the air stream. Air was supplied by a house airline stepped down to 100 ml/min with a flow gauge. The outlet tubing for each flask was a spiral water-trap made from ¼ inch glass tubing topped with a 0.2 micron syringe filter attached by silicone tubing to the vent end of the water trap tubing. The plants were grown in 50 ml medium per flask. Growth temperature was 25 °C.

For cultivation under higher light intensity with diurnal light-dark cycle, cultures of *L. minor* were also grown without perfusion in 100 ml of medium in 32-oz Phytocon™ plant growth containers with lids made of clarified polypropylene (Phytotechnology Laboratories, Kansas, USA) for 1 - 3 months (Fig. 2). These cultures were grown in 1X Schenk and Hildebrandt's basal salts with 0.5 % glucose in 50% D_2O-H_2O and in 100% H_2O. The plants were illuminated with a metal halide lamp in a SunSystem2 fixture at an intensity of 130 μmol m^{-2} s^{-1} with a 12 h light/12 h dark diurnal cycle and temperature of 28 °C.

Plants were harvested by filtration on sterile Miracloth (Calbiochem, La Jolla, CA) and washed with sterile water. Cultures were either immediately frozen and stored at -20 ° C or dried for 2 days at 23 ºC and 21 inches Hg in a vacuum oven. Dry biomass yields from 50 mL cultures

grown under air perfusion and continuous illumination with white fluorescent lamps for 59 d were 0.2514 g for 50% D_2O and 0.0521 g for 60% D_2O. Dry biomass yields from 100 mL cultures inoculated with ten fronds and grown in 32-oz Phytocon™ containers for 38 days were comparable for duckweed grown in H_2O (0.4283 g) and in 50% D_2O (0.4226 g).

Microscopy

Fresh plants were examined under the microscope and photographed at 100 and 400X without decolorization or staining. All images were taken with a Kodak DC290 Zoom Digital Camera using a Kodak MDS microscope attachment with a 7 mm spacer on a Leica Galen III light microscope with a blue filter to normalize coloring through the microscope adapter.

Chlorophyll Assays

No size reduction was necessary for the *Lemna* samples. Chlorophyll was extracted from each sample by addition of 1 ml of methanol. Samples were then briefly vortexed, placed in a 60 ºC water bath for 5 min., vortexed again, and pulse centrifuged at 14,000 rpm for 15 min. The absorbance of the supernatants was measured and then examined with a Cary-Win UV-spectrophotometer at 652 nm, 665 nm, and 750 nm for scatter subtraction. Any samples that gave an absorbance of 2 or greater were diluted with methanol and rescanned. Total chlorophyll content in $\mu g\ mL^{-1}$ was calculated using the coefficients 22.12 and 2.71 at 652 nm and 665 nm for chlorophyll a and b in methanol (Porra 2002, with subtraction of background absorbance at 750 nm according to the equation: $(A_{652} - A_{750})(22.12) + (A_{665} - A_{750})(2.71)$.

NMR Methods

Solid-state NMR was carried out as described previously (Foston et al. 2012). Samples were lyophilized for 4 days to remove residual H_2O and D_2O. Ball-milled samples were loaded into 7-mm cylindrical ceramic MAS rotors. Solid-state NMR measurements were carried out on a Bruker DSX-300 spectrometer operating at frequencies of 46.08 (^2H) and 300.16 (^1H) MHz in a Bruker double-resonance MAS probehead under non-spinning conditions. ^2H NMR spectra were collected using a 90-90 solid-echo sequence for deuterium wide line observation accounting for the detector dead time delay, an echo delay of 50 μs, 16k data points, 250-kHz spectral width, 2-s recycle time, and 2k scans. ^1H NMR spectra were measured with 4k data points, 44-kHz spectral width, 4-s recycle delay and 254 scans. Mixtures of various proportions of natural abundant glucose and glucose-6,6-d_2 were used as standards.

Compositional Analysis

Klason acid-insoluble lignin content was determined by an acid hydrolysis protocol based on Tappi method T-222 om-88 as described previously for annual ryegrass (Evans et al. 2014). The extractive-free samples were delignified using peracetic acid and cellulose was isolated from the delignified sample (holocellulose) by extraction with a 17.5% NaOH solution at 25°C for 2 h. The mixture was diluted to 8.75% NaOH solution by addition of 5 mL of deionized water and repeated stirring at 25°C for an additional 2 h. The isolated α-cellulose samples were then collected by centrifugation, washed with an excess of deionized water and air-dried.

Gel Permeation Chromatography (GPC) analysis of cellulose

Molecular weights of cellulose isolated from *Lemna* grown in 50% D_2O and H_2O-grown controls was determined by gel permeation chromatography (GPC) of the trianthranilate derivatives based on polystyrene calibration standards as described previously (Evans et al. 2014; Evans et al. 2015). The weight-average molecular weights (M_w), defined as the first eluted statistical moment, and the number-average molecular weights (M_n), defined as the second eluted statistical moment, were used to calculate the degree of polymerization (DP) by dividing the M_w by the monomeric unit molecular weight after trianthranilate functionalization. The degree of polydispersity (DPI) was calculated by dividing M_w by the M_n to provide a measure of the range in molecular weights present in a particular cellulose sample.

Results

Comparison of Lemna species minor and gibba

Under the conditions used for cultivation, *Lemna minor* grew faster than *Lemna gibba* in both 50% D_2O (data not shown) and in H_2O solutions of Schenk and Hildebrandt's basal salts (Table 1). Based on these results, *L. minor* was used for subsequent deuterium labeling experiments.

Effects of Carbon Source on Growth of Lemna minor

The substrates acetate, glucose, glycerol, and succinate were initially screened as reduced carbon sources by addition at 0.5% w/v to the culture medium of plants grown under continuous illumination with cool white fluorescent lamps (Fig. 3 and Table 2). They were chosen based on availability in deuterated form and reported assimilation by photosynthetic organisms. As

expected, addition of glucose increased the growth rate. The effect on growth of glucose was determined for concentrations from 0 to 5 %. Optimal growth was observed at 0.5% glucose, while increase to 5% resulted in extreme growth inhibition (Fig. 4). Succinate at 0.5% decreased growth rate to 20% of control, while addition of acetate caused rapid bleaching and death, despite pH adjustment of the stock solutions to pH 4. Further investigation found an $I_{25\%}$ of 0.87 mM for acetate (Fig, 5). Glycerol at 0.5% (54 mM) was inhibitory, reducing growth rate to 4% of controls (Fig. 3 and Table 2).

Perfusion with air from an in-house line supplying compressed ambient air increased the growth rate of *L. minor* in medium supplemented with 0.5% glucose. For *L. minor*, initial growth rate was increased from 3.6 to 7.5 fronds d^{-1}, while linear growth rate after 10 days post inoculation was increased from 21.5 to 32.5 fronds d^{-1} (Table 1).

Effects of Deuteration on Growth and Morphology

Growth of *L. minor* was screened for D_2O concentrations of 50, 60, and 70% (Fig. 6 and 7). As had been reported previously for *Lemna perpuspilla* (Scope et al. 1965), growth rate in 50% D_2O was 40% of that in natural abundance water, while further increase to 60 and 70% D_2O resulted in a precipitous drop in growth and increased mortality. Similar results were observed for the terrestrial monocot annual rye grass (Evans et al. 2014). Production of deuterium-labeled biomass was therefore carried out in 50% D_2O with 0.5% glucose supplementation.

The initial tests of D_2O tolerance using continuous illumination with white fluorescent lamps found that, after a lag phase of approximately ten days, *L. minor* started to grow in 50 and 60% D_2O at rates one-half and one-third, respectively, of those of control plants (Fig. 7). Under more intense illumination in a diurnal cycle of 12 h light/12 h dark, the lag phase before onset of exponential growth in 50% D_2O was approximately 20 days for growth in 50% D_2O and 50 mM glucose (Fig. 8; Table 3). The increased illumination intensity and wavelength range of the metal halide lamp induced a two-fold increase in the log phase growth rate of *L. minor* in 50% D_2O compared to five-fold increase for the control in H_2O media. The 50% D_2O growth rate in both initial (lag) phase and log phase growth was approximately 40% of that measured for the control duckweed grown in H_2O, similar to the reduction in growth rates observed previously for the terrestrial monocot annual ryegrass (Evans et al. 2014). The yields of dry biomass at 38 days were found to be comparable for the H_2O and D_2O cultures despite the slower growth initial and log-phase growth rates in 50% D_2O. Once the cultures have reached confluence, the growth rates can be expected to slow due to crowding and shading.

Inhibition of root elongation by 50% D_2O was observed for *L. minor* (Fig. 9 and Table 5), consistent with results reported by earlier studies (Cope et al. 1965; Trewavas 1970). Control plants grew roots with average length 1.605 ± 0.904 cm, while plants grown in 50% D_2O had an average root length of 0.31 ± 0.16 cm ($P < 0.00001$). In contrast to the increase in average frond surface area reported for *L. perpusilla* grown in 50% D_2O with protiated glucose (Cope et al. 1965), fronds of *L. minor* were slightly smaller but heavier than those of controls grown in H_2O (Table 5). Examination of cellular morphology under the light microscope (Fig.10) did not detect any changes in cell wall dimensions or appearance in the fronds.

Biomass yields calculated as mg mL^{-1} d^{-1} of duckweed grown in 50% D_2O were increased by 30% when cultures were grown at the higher light intensity under a diurnal cycle of 12 h light/12 h dark.

Deuterium Incorporation

According to the results from the solid-state NMR analysis, deuterium substitution levels of 40 – 50 % were achieved by cultivation in media containing 50% D_2O and 0.50 % glucose (Table 4). This level of partitioning of deuterium label from D_2O in the growth media in the presence of a hexose carbon source is consistent with earlier studies of *Lemna* species grown photoheterotrophically in 50 and 60% D_2O with glucose (Cope et al. 1965).

Compositional Characterization

The degree of polymerization (DP) and polydispersity index (DPI), are parameters used to compare polymers such as cellulose, hemicellulose, and lignin from different sources (Foston and Ragauskas 2010), between deuterated and control bacterial cellulose (Bali et al. 2013), and between deuterated and control plants (Evans et al. 2014; Evans et al. 2015). Cellulose isolated from *L. gibba* plants grown in 50% D_2O had a substantially lower DP_w that was 63% of that of controls grown in H_2O. The DP_W of cellulose isolated from *L. minor* grown in 50% D_2O was 85% of the DP_w of H_2O-grown controls (Table 5). This is consistent with the results reported previously for the terrestrial species annual ryegrass (Evans et al. 2014) and switchgrass (Evans et al. 2015), which found that cellulose isolated from plants grown in 50% D_2O had DP close to that of control plants grown in H_2O.

Determination of Klason lignin found that growth in 50% D_2O decreased lignin content from 18 to 8 % dry weight (Table 7). Previously published studies have generally found the lignin content of *Lemna* duckweed species to be much lower. When the low yields of about 10% for the alkaline cupric hydroxide method are taken into account, a content of around 2.5% derived from H and G units can be estimated from previously reported determinations (Blazey and McClure, 1968).

Those results are consistent with the lignin content of 2.4% dry weight determined as Klason lignin for *L. minor* harvested from the wild in Great Britain reported later (Zhao et al. 2014). In a report surveying the phenolic constituents of the Lemnaceae, *L. minor* lignin was once again reported to be composed of *p*-coumaryl and coniferyl (H and G) units (McClure 1975). However, a Klason lignin content of 12% was reported for *Lemna perpusilla* collected in Calcutta, India (Chanda et al. 1991), indicating possible variance in lignin content dependent on purification protocols, assay choice, and growth conditions. The lower lignin content of *L. minor* grown in 50% D_2O resembles the results reported earlier for annual ryegrass (Evans et al. 2014), while switchgrass grown hydroponically in 50% D_2O-exhibited higher lignin content than H_2O-grown hydroponic switchgrass (Evans et al. 2015).

Discussion

The growth inhibition observed for supplementation with 0.5% glycerol (approximately 54 mM) could be due to sensitivity of the duckweed to osmotic stress induced by polyols. Mannitol, a six-carbon polyol, is used at concentrations of 100 – 400 mM to induce osmotic stress in plant experiments (Butt et al. 2017; Singh et al. 2015). A standard method for assay of drought tolerance utilizes 5% polyethylene glycol 6000 (Joshi et al. 2017). Sodium acetate was found to be rapidly toxic to *L. minor* with an $I_{25\%}$ of 0.89 mM. As salinity from sodium chloride has been reported to inhibit growth of *L. minor* at concentrations greater than 25 mM over the course of three days, it appears unlikely that the sodium counter ions were responsible for this rapid toxic effect (Liu et al. 2017).

Inhibition of root elongation by 50% D_2O in *Lemna* is consistent with earlier studies for this genus as well as terrestrial plants. In this study, common duckweed *Lemna minor* was found to adapt better to growth in 50% D_2O than *L. gibba* based on the growth rates and the properties of the isolated cellulose. Cellulose isolated from *L. gibba* had a DP_w more than twice that of *L. minor* cellulose. The differences in cellulose chain length may be correlated with the morphology of *L. gibba* which is distinguished from that of *L. minor* by larger frond size and presence of a vascular structure called a nerve. Both species exhibited cellulose PDI values, whether grown in H_2O or in 50% D_2O, approximately three-fold higher than those determined for annual ryegrass and for switchgrass grown under similar conditions (Evans et al. 2014; Evans et al. 2015),

Analysis of sequentially extracted fractions of *Lemna minor* biomass found a composition typical of primary cell walls, being largely composed of cellulose and pectin, with relatively small amounts (around 3%) of hemicellulose and lignin (Xhao et al. 2014). Celluloses extracted from primary cell walls are reported to have average degrees of polymerization in the range of 2,000 – 6,000 glucose residues, while secondary cell walls contain longer cellulose molecules with DPs as high as 10,000 (Reid et al. 1997).

The phenomenon of growth inhibition by D_2O at 50% and higher is likely to be the cumulative result of specific impacts on multiple metabolic pathways. A correlation between germination and growth with cold tolerance and the differences in the physical properties of D_2O (higher viscosity, higher melting point, and higher temperature of maximum density) had been noted in earlier studies (Siegel et al. 1964; Blake et al. 1968). Both growth rate and metabolism of *L. minor* were observed to change in response to water temperature. Within a temperature range of 8 – 31 °C, temperatures lower than 25 °C result in slower growth and higher ratios of carbohydrate to protein in the plant biomass (Bornkamm 1968). Increase in growth temperature may improve growth in 50% D_2O, as had been reported for winter grain rye (Siegel et al., 1974). The membrane potential of *Lemna* species increases in response to light, believed to be mediated by phytochrome (Löppert et al. 1978) and to hexoses in the media (Novacky et al. 1978). Both uptake of hexoses and amino acids from growth media have been shown to be coupled to proton transport. *Lemna* species are reported to grow in the pH range of 4.5 – 7.2 (Stomp, 2005). Previously reported studies of cultivation of *Lemna spp*. in 50% D_2O have used media at pH 5.0, observing reduced growth rate and shortened roots (Trewavas 1970).The medium formulation published by Hutner, with a pH approximately 4.8, as well as Hoagland's medium with pH were used in studies of *Lemna perpusilla* in 50 – 63% D_2O (Scope et al. 1965), while Cooke and co-workers (Cooke et al. 1979; Cooke et al. 1980) used the medium published by Trewavas (1970). The lower pH of 4.2 employed in this study may have partially ameliorated the decrease in membrane potential due to the effects of 50% D_2O, which can be expected to include both 50% slower transport of deuterons compared to protons (De Coursey and Cherny 1997) as well as the disturbance of the phytochrome equilibrium (Sarkar and Song 1981; Borucki et al. 2005). Differences in results from these reported studies may also stem from variation in the type of illumination used for cultivation. Inhibition of *L. minor* by phenylalanine was previously shown to be correlated with intensity and spectral characteristics of illumination (Evans et al. 2017).

Growth in 50% D_2O had been previously reported to result in morphological changes to the cellular structure of fronds of *Lemna perpusilla* (Cope et al. 1965). Enlargement of cells, decrease in size of air spaces, and disorganization of cellular arrangement in frond tissues were visible at 150X magnification. Changes to the ultrastructure of the tonoplast and chloroplast membranes of *Lemna minor* during initial exposure to 50% D_2O, followed by recovery and adaptation after 24 h, were observed by electron microscopy (Cooke et al. 1980). Protein fractions from the isotopically stressed *L. minor* were more susceptible to protease digestion, similar to those from nitrate-stressed plants. However, examination of seedlings of winter rye (*Secale cereale*) germinated in 99.8% D_2O by electron microscopy found no major differences in the cellular ultrastructure compared to H_2O-germinated controls (Waber and Sakai 1974).

The decrease in the yield of Klason lignin determined for the duckweed grown in 50% D_2O could result from the known kinetic isotope effects of D_2O on phytochrome equilibria (Sarkar and Song 1981). The enzyme phenylalanine ammonia lyase (PAL) is known to be induced through a

phytochrome-activated pathway. *L. minor* produces PAL and tyrosine ammonia lyase (TAL) in synchrony with the light cycle used for cultivation, and induction by illumination with red light has been demonstrated. Photoactivation and cycling of phytochromes have been shown to be perturbed by D_2O with relatively large solvent kinetic isotope effects (Sarkar and Song 1981).

In conclusion, the results of this study indicate that production of 40 – 50% deuterated biomass can be carried out by cultivation of *Lemna minor* duckweed at increased growth rates under higher illumination levels with diurnal period. Plants eventually adapt to growth in 50% D_2O without addition of growth hormones but continue to exhibit root stunting and slower growth rates than H_2O-grown controls. Cell wall appearance and cellulose degree of polymerization resembled those of H_2O-grown controls, indicating that duckweed could be used to produce deuterium-enriched carbohydrates.

Author Contribution Statement

BRE carried out plant cultivation experiments with assistance of DTR, CSR, KMcG, and HO'N. MF and GB carried out characterization by NMR, FTIR, and other methods at Georgia Tech under supervision of AJR. BD coordinated and led the research project.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Table 1 Effects of air perfusion and glucose supplementation on growth were compared for the duckweed species *Lemna gibba* and *Lemna minor* grown under continuous white fluorescent light

Species	Air perfusion (ml min^{-1})	Glucose concentration (%)	Growth Rate (fronds d^{-1})
Lemna gibba	0	0	2.9
Lemna gibba	0	0.5	26
Lemna gibba	100	0	3.1
Lemna gibba	100	0.5	12
Lemna minor	0	0	3.6
Lemna minor	0	0.5	24
Lemna minor	100	0	6.3
Lemna minor	100	0.5	38

Table 2 Growth of *Lemna minor* was compared for potential fixed carbon sources available in deuterated form at 0.5% w/v under continuous illumination with white fluorescent light. Rates were determined by best fit with linear regression of the data presented in Figure 3 for days 7 to 19

Fixed Carbon Source	Growth Rate (fronds d^{-1})	%I (days 1-7)	%I (days 1-14)
None	26.1	NA	NA
Acetate	0	100	100

Glucose	29.5	0	-26
Glycerol	5.86	5.6	61
Succinate	13.1	0	32

Table 3 Initial and log-phase growth rates of *Lemna minor* grown with 0.5% glucose in H_2O and in 50% D_2O under illumination with a metal halide lamp and diurnal cycle of 12 h light/12 h dark were compared. Growth rates were determined by best fit linear regression from the data presented in Figure 8

Growth Solution	Growth Rates (fronds d^{-1})	
	Initial	Log-phase
H_2O	16.2	150.6
50% D_2O	5.57	59.7

Table 4 Deuterium incorporation in whole dried *L. minor* grown in Schenk and Hildebrandt's basal salts in 50% D_2O was determined by ^1H ^2H-solid phase NMR

Species	% D_2O in Medium	Growth time (d)	Deuterium Content (% dry wt)
Lemna minor	50	59	57.5 ± 13.3
Lemna minor	50	41	45.8 ± 7.4

Table 5 Gross morphology and chlorophyll content of *L. minor* grown in 50% D_2O was compared to those of H_2O-grown controls

Growth Medium	H_2O	50% D_2O
Average Frond Size (mm) Length Width	 2.8 ± 0.14 (n=147) 2.3 ± 0.10 (n=147)	 2.5 ± 0.10 (n=174) 1.9 ± 0.07 (n=174)
Average Root Length (cm)*	1.6 ± 0.40 (n=20)	0.31 ± 0.07 (n=20)
Average Weight of 35 fronds** (g)	0.0271 ± 0.0011 (n=4)	0.0308 ± 0.0050 (n=5)
Average Chlorophyll Content of 35 fronds (% total weight)***	0.1132 ± 0.0087 (n=4)	0.0654 ± 0.0300 (n=4)

*P < 0.00001; **P > 0.1; ***P < 0.05

Table 6 Degree of polymerization of cellulose (DP) isolated from *L. minor* and *L. gibba* duckweed grown in H_2O and in 50% D_2O was calculated from the number average molecular weights (M_n) and weight average molecular weights (M_w) determined by gel permeation chromatography. The polydispersity index (PDI) was calculated by dividing DP_w by DP_n

Species	Growth Medium	DP_n	DP_w	PDI
Lemna gibba	50% D_2O	278	1765	6.35
Lemna gibba	H_2O	440	4018	9.13
Lemna minor	50% D_2O	146	1126	7.71
Lemna minor	H_2O	171	1861	10.9

Table 7. Lignin content of *Lemna minor* grown in 50% D_2O and in H_2O was determined as percent Klaason lignin per dry weight

Species	Growth Medium	Lignin Content (%)
Lemna minor	50% D_2O	8
Lemna minor	H_2O	18

Figure Legends

Fig. 1 A perfusion system (front row of flasks) with water-trap condensation coils was used for initial studies of cultivation of the duckweed *Lemna minor* in D_2O-H_2O mixtures under continuous illumination with white fluorescent light

Fig. 2 For scaled up biomass production, *L. minor* was cultivated in plant growth containers without perfusion in 1X Schenk and Hildebrandt's basal salts with 0.5% glucose under illumination with a metal halide lamp and diurnal cycle of 12 h light/12 h dark. Left, H_2O control; right, 50% D_2O

Fig. 3 Effect of carbon sources at 0.5% on growth of *L. minor* was evaluated for cultures grown under continuous illumination with white fluorescent light

Fig. 4 The optimal concentration of glucose for growth of *L. minor* under continuous white fluorescent light was determined to be 0.5% (27.7 mM)

Fig. 5 The inhibitory effect of acetate was further investigated in *Lemna minor* cultures illuminated in 12 h light/12 h dark diurnal cycle with Gro-Lux lamps and the $I_{25\%}$ was determined to be 0.87 mM

Fig. 6 The growth of *L. minor* in H_2O-D_2O mixtures was examined for periods of 10 – 50 days to determine the optimal concentration for the deuteration experiments

Fig. 7 Comparison of growth rates of *L. minor* in H_2O-D_2O mixtures determined from the data presented in Figure 5 showed that growth rates dropped precipitously beyond 50% D_2O

Fig. 8 Initial and log-phase growth rates were compared for *L. minor* grown in plant growth containers in H_2O and in 50% D_2O under illumination with a metal halide lamp and a diurnal cycle of 12 h light/12 h dark. Growth media were supplemented with 0.5% glucose. The rates determined by linear regression fitting are presented in Table 3

Fig. 9 Roots of *L. minor* plants grown in 50% D_2O were shorter than those of control plants grown in H_2O. Glucose concentration in the media was 0.5%

Fig. 10 Examination of *L. minor* fronds under the light microscope found the cellular morphology of plants grown in H_2O and in 50% D_2O to be similar with no obvious abnormalities. Control plants grown in H_2O at 100X (A) and 400X (B); plants grown in 50% D_2O at 100X (C) and at 400X (D)

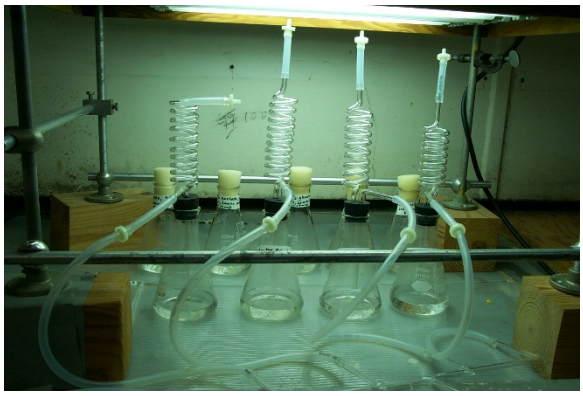
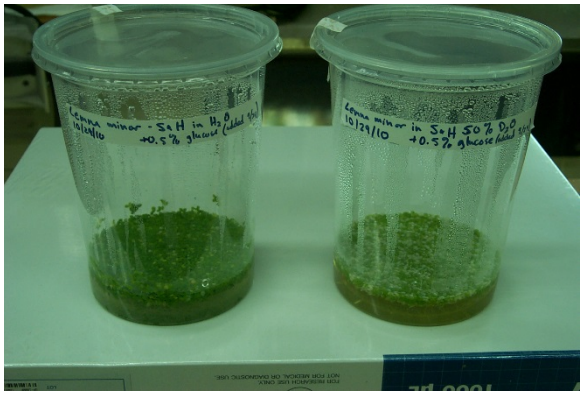


Fig. 1



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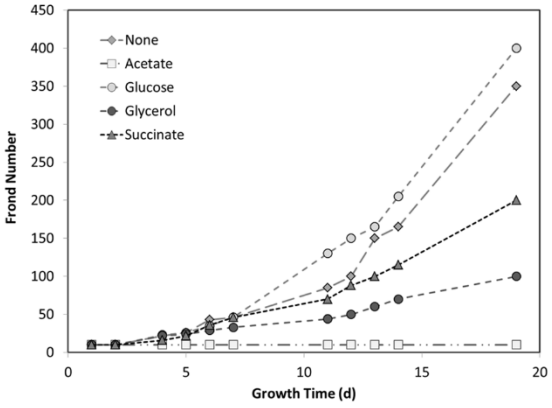

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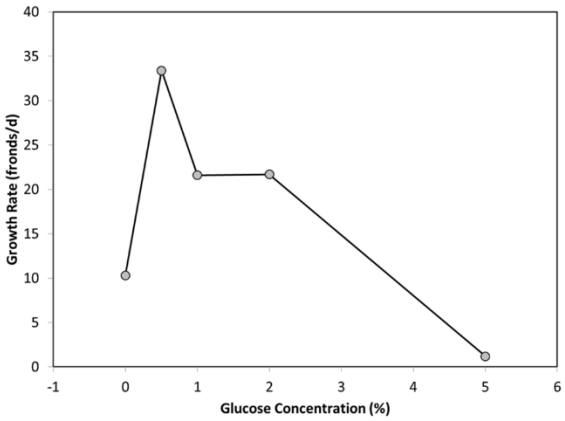

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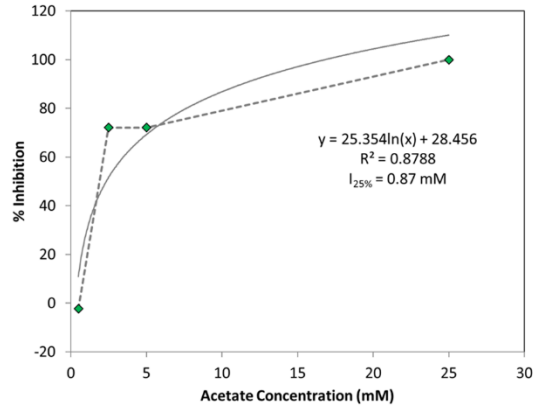

Fig. 5

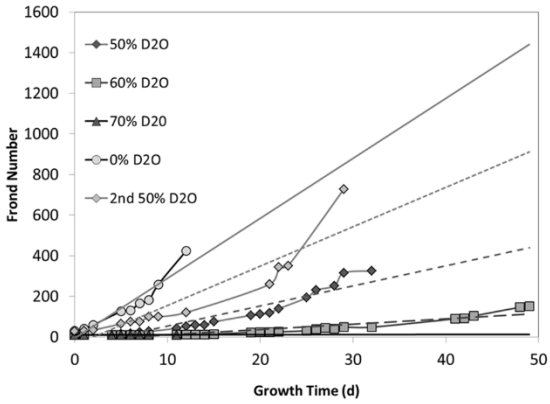

Fig. 6

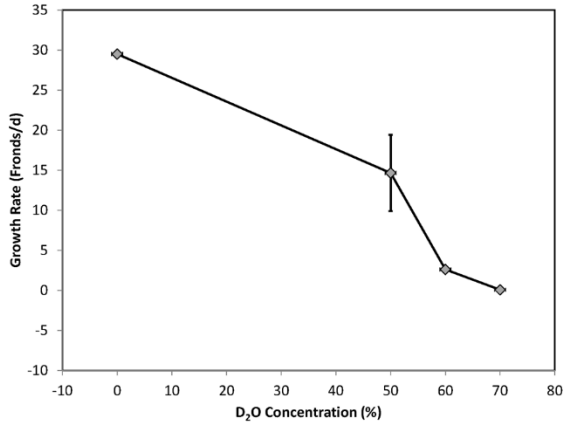

Fig. 7

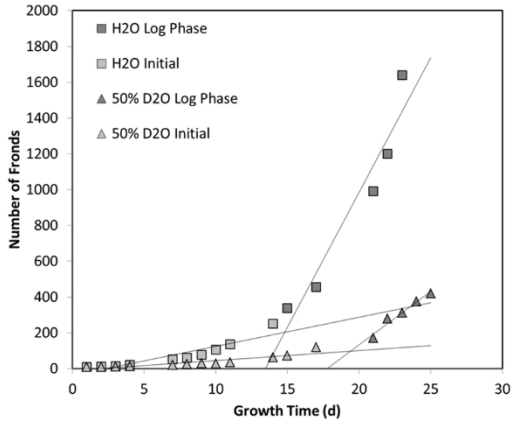

Fig. 8

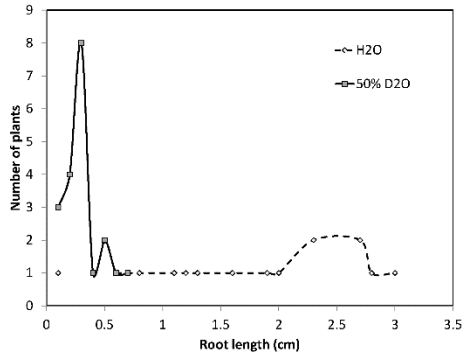

Fig. 9

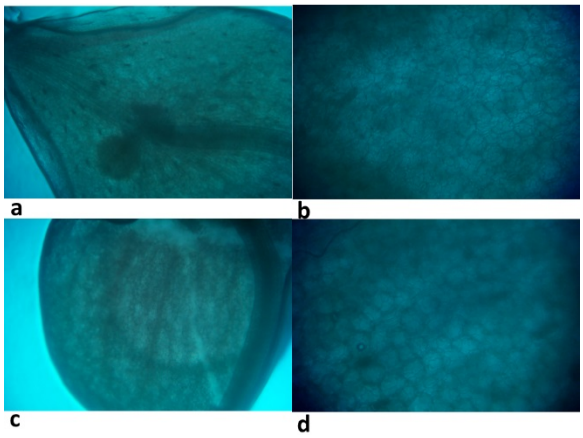

Fig. 10