Algal Research 15 (2016) 152–163

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

# Cyanobacterial and algal growth on chitin as a source of nitrogen; ecological, evolutionary, and biotechnological implications☆

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ARTICLE INFO

*Article history:*
Received 13 August 2015
Received in revised form 8 January 2016
Accepted 10 February 2016
Available online xxxx

*Keywords:*
Chitin
Nitrogen metabolism
Lipid hyperaccumulation
Oil
Secondary carotenoids
Renewable biomass production

ABSTRACT

Chitin is the second-most abundant natural polymer and a waste product of the shellfish industry. We isolated more than 60 different strains of cyanobacteria and microalgae capable of using chitin as a sole source of nitrogen. Eleven axenic algal strains still retain the ability to grow on chitin and/or chitosan, and specific growth rates are comparable to rates on conventional sources of nitrogen. 16S rDNA shows that chitin utilization in phototrophs is ecologically widespread, revealing a new connection between light and the biogeochemical cycling of carbon and nitrogen. Most cyanobacterial strains in the UMPCCC (University of Montana Photosynthetic Chitin Culture Collection) fall into four clades: the *Cyanobia*, *Leptolyngbyas*, *Oscillatorias*, and *Nodularias*. Microalgal taxa in the collection fall into the Chlorophyceae, Trebouxiophyceae, Eustigmatophyceae, and Bacillariophyta, including the commercially important genera *Chlorella*, *Chlamydomonas*, *Haematococcus*, and *Dunaliella*.
This work shows that Chlorophyta grown on chitin have decreased chlorophyll content, and some taxa exhibit secondary carotenogenesis, the production of orange and orange-red pigments, during the late exponential and early stationary phases. Six (eukaryotic) microalgal strains tested exhibited lipid hyperaccumulation on chitin. All showed a 1.5–5-fold increase in oil content (measured as pg of oil content per cell). Lipid hyperaccumulation and secondary carotenogenesis did not require nitrogen starvation or complex treatment of the cells. Thus, microalgal growth on chitin may provide an efficient method for the production of natural pigments and algal biofuels. Finally, we show that chitin has the potential to reduce the greenhouse gas footprint for algal biomass production, making algal biofuels and bio-products more sustainable and economical. It also opens a new avenue for algal-based wastewater treatment using an insoluble form of nitrogen. This reveals a new, renewable nitrogen source for the mass cultivation of photosynthetic biomass, turning a waste product into valuable commodities.



## 1. Introduction

Nitrogen metabolism has been well studied in the cyanobacteria and algae [1,2]. Naturally occurring sources of inorganic nitrogen that support the growth of oxygenic phototrophs include nitrate, nitrite, and ammonia [3]. Ammonium is generally considered to be a preferred substrate, since it is the product of nitrogen fixation, is the dominant source of nitrogen in the oceans, and nitrate is reduced to ammonium prior to assimilation into biomass, however inorganic nitrogen preferences are often species-specific [2,4]. Dinitrogen can be assimilated by nitrogen-fixing cyanobacteria, where nitrogen gas is reduced to ammonium [5,6]. Naturally occurring sources of organic nitrogen that support the growth of oxygenic phototrophs include urea, cyanate, purines, hypoxanthine, xanthine, urate, amino acids, and oligopeptides [7–9]. Urea assimilation requires uptake and reduction to ammonium by urease or urea amidolyase [9]. For assimilation of cyanate (a breakdown-product of urea in the aqueous environment), cyanase converts cyanate to carbon dioxide and ammonia [10]. Amino acids and other sources of organic nitrogen can be used by some individual phytoplankton species [11].

Chitin is an organic, nitrogen-containing, water insoluble, long-chain polymer of N-acetylglucosamine. It is found in crustacean exoskeletons, insects, mollusks, and the cell walls of fungi and some unicellular eukaryotes. One of the most abundant biopolymers on the planet, it has an estimated natural production of $10^{11}$ metric tons per year [12]. Chitin is a waste product of the shellfish industry. In the United States, 750,000,000 lb of crab, shrimp, and lobster is harvested annually [13]. Crabs make up 47% of the catch, lobsters 16%, and shrimp 35%. Once the meat is removed, the shells are often sent to the landfill. The chitin content of the shells varies from 10–40% [14–18].

Chitin is also an important source of N and C in aquatic environments where it is efficiently degraded by microbial processes involving chitinase, chitosanase, and chitin deacetylase enzymes [19,20]. Multiple

*Abbreviations:* GH, glycosyl hydrolase; UMPCCC, University of Montana Photosynthetic Chitin Culture Collection.

☆ Data deposition: The sequences reported in this paper have been deposited in GenBank (accession nos. KM218863–KM218906)

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http://dx.doi.org/10.1016/j.algal.2016.02.014
2211-9264/

convergent pathways have evolved to metabolize chitin [21]. All known chitin-catabolizing microorganisms have been heterotrophic bacteria and archaea [22]. Hence, the observation of putative chitinases in several cyanobacterial genomes [23] was unexpected. This prompted us to set up enrichment cultures for phototrophic microorganisms capable of growing on chitin as a sole source of nitrogen.

We show here that 29 strains of cyanobacteria and 32 strains of eukaryotic microalgae are capable of growth on chitin as a sole source of nitrogen. The aims of this work were to study the growth dynamics of algal growth, by comparing the growth rates on chitin with growth rates on conventional sources of nitrogen (nitrate, ammonia, urea). Another aim was to determine whether bacteria were necessary for the algal cultures to grow on chitin, thus eleven microalgal strains were rendered axenic (free of heterotrophic bacteria). Another aim was to examine the phylogenetic distribution of cyanobacteria and microalgal growth on chitin, thus 16S rDNA was obtained and phylogenetic trees were constructed. Another aim was to examine the lipid content of chitin-grown microalgae, thus Nile Red staining and gravimetric oil determinations were done on parallel cultures grown on chitin and ammonia (or nitrate). Finally, five Chlorophyta cultures were observed to exhibit different pigment characteristics when grown on chitin. Therefore, simple organic extractions followed by spectrophotometric analyses were performed to quantify chlorophyll and carotenoid contents of microalgal biomass grown on chitin, ammonium, and nitrate. Based on the results we show, the biotechnological implications for using a renewable source of nitrogen that is a waste product of the shellfish industry to make a valuable commodity (algal biomass) are discussed. Finally, we suggest that the use of chitin as a source of nitrogen could potentially reduce the carbon footprint of algal biomass production.

## 2. Materials and methods

### 2.1. Enrichment cultures

Details on enrichment culture methodology can be found in Blank and Hinman [23]. Nitrogen-free medium was prepared and purified; ground, autoclaved chitin was added (to a final concentration of 1–3 g $L^{-1}$). Nitrogen-free versions of culture media included a modified nitrogen-free BG-11 (two pH versions: pH 7.0 and pH 4.3), a nitrogen-free version of ASNIII, and Soap Lake Autotrophic Medium, a unique alkaline, brackish medium designed to mimic the geochemistry of Soap Lake, WA (for recipe see reference []). The modified, nitrogen-free BG-11 medium contained (per liter): 0.04 g $K_2HPO_4$, 0.075 g $MgSO_4 \times 7H_2O$, 0.036 g $CaCl_2 \times 2H_2O$, 0.02 g $Na_2CO_3$, 2.5 mL nitrogen-free iron solution, 1 mL trace metal mix, and EDTA (5 mM final concentration). The nitrogen-free iron solution contained (per liter): 270 mg ferric citrate, 590 mg sodium citrate, and HCl (1.2 mN final concentration). The trace metal mix contained (per liter): 2.86 g $H_3BO_3$, 1.81 g $MnCl_2 \times 4H_2O$, 0.222 g $ZnSO_4 \times 7H_2O$, 0.39 g $NaMoO_4 \times 2H_2O$, 0.079 g $CuSO_4 \times 5H_2O$, and 0.049 g $CoCl_2 \times 6H_2O$. The pH was then adjusted to either 7.0 or 4.3 with HCl. The modified N-free ASNIII medium contained (per liter): 75 g NaCl, 3.5 g $MgSO_4 \times 7H_2O$, 2 g $MgCl_2 \times 6H_2O$, 0.5 g $CaCl_2 \times 2H_2O$, 0.375 g KCl, 2.25 g $K_2HPO_4$, 3 mL trace metal mix, 7.5 mL nitrogen-free iron solution, and EDTA (5 mM final concentration). The pH was adjusted using concentrated KOH to 8.1–8.2.

Inocula derived from natural sources of water or surface sediment (see Table S1 for sample type, sample location, and GPS coordinates of each sample location), and (liquid batch suspension) enrichment cultures containing chitin (either 75 mL of culture medium in 250 mL Erlenmeyer flasks or 50 mL liquid cultures in 125 mL Erlenmeyer flasks) were incubated under continuous (24-hour illumination) fluorescent lighting (Philips Natural Light T8 lamps, at an irradiance of ~54 μmol photons $m^{-2}$ $s^{-1}$) until pigmented photosynthetic organisms appeared (ranging anywhere from 1–3 weeks). Enrichment cultures were not aerated mechanically. The inoculum volume was approximately 1:100 the enrichment culture volume. Unialgal cultures were obtained through repeated serial dilution on culture medium containing inorganic nitrogen (nitrate or ammonia) to reduce the diversity and abundance of heterotrophic organisms. The purity of the cultures was then determined using phase-contrast microscopy and 16S rDNA analyses (below). Some unialgal cultures (*Dunaliella* sp. UMPCCC 1206 and *Leptolyngbya* sp. UMPCCC 1225) were obtained by streaking onto agar plates containing inorganic nitrogen. Isolated, pigmented colonies were then inoculated into liquid cultures, and purity of the cultures verified using phase-contrast microscopy and 16S rDNA analyses. All unialgal cultures were subsequently maintained on inorganic nitrogen. Cultures were maintained as liquid batch suspension cultures in 125 mL and 250 mL borosilicate Erlenmeyer flasks (with 50–75 mL culture volumes to facilitate gas exchange). Flasks were closed with sterile cotton plugs wrapped in cheesecloth, allowing diffusion of atmospheric gases in and out of the flask, and were not aerated mechanically. Cultures were stationary and occasionally mixed by hand (every day or two). Cultures were routinely maintained at room temperature under aerobic conditions and continuous (24-h) illumination, using full-spectrum fluorescent lights. Sampling locations for cultures in the UMPCCC (University of Montana Photosynthetic Chitin Culture Collection) are listed in Table S1.

### 2.2. Nitrogen testing

Eleven algal strains (listed in Table S2) were rendered axenic by serially streaking on agar plates containing inorganic nitrogen until single colonies isolated from any heterotrophic colonies were obtained. Next, the axenic status of the strains was verified by performing three serial passages on culture medium containing chitin, chitosan, and medium supplemented with 10% Luria-Broth (containing yeast extract and Bacto-Tryptone). The absence of bacteria was confirmed using phase-contrast microscopy (at 400× magnification).

The relative nitrogen affinities of 45 of the unialgal isolates (listed in Table S3), spanning the evolutionary diversity of the strains in the collection, were determined by at least three serial passages on a single nitrogen source (sodium nitrate, 588 μM; ammonium chloride, 500 μM; urea, 500 μM; or chitin, 3% wt./vol.). The relative nitrogen affinities of all 11 axenic strains were determined by three serial passages on a broader diversity of nitrogen compounds (also including N-acetylglucosamine, 100 μM; glucosamine, 100 μM; or chitosan, 3% wt./vol.). Nitrogen testing was carried out in 3 mL culture volumes in 18 × 150 mm borosilicate glass culture tubes (standing cultures incubated at room temperature, mixed manually every day or two, under 24 hour illumination). Cultures were transferred after 3 weeks of incubation.

Eleven cyanobacterial cultures (listed in Table S4) were incubated under aerobic N-free culture conditions to test for the ability to fix nitrogen. Cultures selected for testing were those that were members of clades that have taxa with genomes that contain inferred nitrogenase genes (in the LPP-B clade strains tested were UMPCCC 1102, 1105, 1225, 1227, and 1237; in the PNT clade they were UMPCCC 1106, 1112, 1204, and 1211; in the SPM clade they were UMPCCC 1104 and 1209). Tested cultures were grown under continuous illumination until they reached the stationary phase (3 weeks). Growth was quantified and measured using a phase-contrast hemocytometer (see below). Abundant growth on chitin, with little or no growth in nitrogen-free medium held under the same environmental conditions, was interpreted as growth on chitin that could not be attributed to nitrogen fixation, however this is a speculation and needs to be independently verified. The presence of nitrogenase genes in related cyanobacterial genomes was determined using PBLAST or tBLASTn using a known, genetically identified nitrogenase sequence (NifH) from *Leptolyngbya boryana* (accession no. AB808482.1; reference [24]). A positive BLAST result was designated by a BLAST score that was $<1e^{-40}$. The genomes analyzed were those in the tree in Fig. 1. The number of related genomes with NifH homologs