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Magnetic separation of algae genetically modified for increased intracellular iron uptake

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ABSTRACT

Algae were investigated in the past as a potential source of biofuel and other useful chemical derivatives. Magnetic separation of algae by iron oxide nanoparticle binding to cells has been proposed by others for dewatering of cellular mass prior to lipid extraction. We have investigated feasibility of magnetic separation based on the presence of natural iron stores in the cell, such as the ferritin in Auxenochlorella prototothecoides (A. protothecoides) strains. The A. protothecoides cell constructs were tested for inserted genes and for increased intracellular iron concentration by inductively coupled plasma atomic absorption (ICP–AA). They were grown in Sueoka's modified high salt media with added vitamin B1 and increasing concentration of soluble iron compound (FeCl$_3$ EDTA, from $1 \times$ to $8 \times$ compared to baseline). The cell magnetic separation conditions were tested using a thin rectangular flow channel pressed against interpolar gaps of a permanent magnet forming a separation system of a well-defined fluid flow and magnetic fringing field geometry (up to 2.2 T and 1000 T/m) dubbed "magnetic deposition microscopy", or MDM. The presence of magnetic cells in suspension was detected by formation of characteristic deposition bands at the edges of the magnet interpolar gaps, amenable to optical scanning and microscopic examination. The results demonstrated increasing cellular Fe uptake with increasing Fe concentration in the culture media in wild type strain and in selected genetically-modified constructs, leading to magnetic separation without magnetic particle binding. The throughput in this study is not sufficient for an economical scale harvest.

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1. Introduction

The United States Energy Information Administration estimates that the world energy need will increase to more than 800 quadrillion BTU by 2040, a 56% energy increase since 2010 [1]. The leading energy consumption is from non-renewable resources, further exacerbating the looming energy crisis. Algae are one possible option as renewable biofuel. Microalgae can produce a high oil yield up to 15.5 L per 1 m^2 aquaculture surface area per year [2]. Converting algae to biofuel is accomplished in stages, including an all-important step of biomass dewatering by $\sim 100 \times$ necessitated by a relatively low algae mass concentration of 0.5 g/L. In the final stage the oil is retrieved from the algae in a

number of ways, including pressing, chemical extraction, and enzymatic extraction [3].

This paper focuses on the feasibility of concentrating, or dewatering, of the algae by magnetic means. Magnetic separation of algae has been proposed based on iron oxide nanoparticle binding to cells [4]. The label-less magnetic separation of magnetotactic algae has been also proposed by others [5]. Eliminating the need for magnetic particle attachment reduces the number of steps in the algae to biofuel process, eliminates the contamination by iron oxide nanoparticles and thus, in principle, could reduce the production cost. The feasibility of magnetophoresis and magnetic separation based on cell intrinsic magnetic susceptibility has been demonstrated before for intraerythrocytic malaria parasite detection [6] and for selected cancer cell lines analysis [7].

A strain of Auxenochlorella prototothecoides (A. protothecoides), KRT1006, was genetically modified by Phycal Inc. to yield three strains of increased iron scavenging ability and magnetic susceptibility. These were used for the magnetic separation studies. The

magnetic separation conditions were tested using a small scale magnetic deposition microscopy (MDM) device [8–10] and compared to theoretical deposition model of non-interacting particles of known magnetophoretic mobility entrained in a laminar flow of viscous media, at low particle volume concentration of less than 1%, applicable to this study [11]. Both genetic modifications and the soluble iron compound concentration in the growth media were considered in the experimental plan.

2. Materials and methods

2.1. Algae preparations

Algal strains engineered with enhanced iron transport and storage capabilities were developed with KRT1006, a strain of *A. protothecoides* (*A. protothecoides*) (by Phycal Inc., Highland Heights, OH). Three target genes were identified which are involved in mediating iron homeostasis. Iron assimilation protein (Fea1 protein) is a high affinity Fe^{2+} transporter native to *Chlamydomonas reinhardtii* that is well characterized and is known to selectively transport iron [12–15]. This reduces the risk of accumulating other heavy metals which may become toxic to the algae. An associated ferrireductase protein (Fre1 protein) acts as an iron reductase ($Fe^{3+} \rightarrow Fe^{2+}$) that is also membrane associated and exposed to the periplasmic space between the outer cell membrane and the cell wall. It is suggested that Fre1 reduces iron so that it is available for transport by Fea1. Both of their respective genes are known to be induced under low iron conditions. The third target gene encodes the Fer1 or ferritin protein. Ferritin and bacterioferritin are highly conserved across a broad range of organisms and play a significant role in iron homeostasis [16]. Ferritin and bacterioferritin store iron in the form of ferrihydrite inside their central cores [17]. The iron stored in the ferritin complex can be retrieved for later use by the cell when iron becomes limiting. Ferrihydrite is reported to be paramagnetic [18].

A large number of *A. protothecoides* clones were developed with enhanced assimilation, storage and tolerance to paramagnetic elements. Nuclear transformation of *A. protothecoides* with the vector pP0176 carrying *Fer1* and *Fea1* genes, and vector pP0175 carrying *Fre1* was performed by simultaneously bombarding these vectors into *A. protothecoides* [12,14] Transgenic clones were initially selected for their resistance to hygromycin. The hygromycin resistant transformants were then transferred to media containing the antibiotic paromomycin to test their resistance. *A. protothecoides* transformants exhibiting resistance to both hygromycin (coming from vector pP0176 carrying *Fer1* and *Fea1*) and paromomycin (coming from vector pP0175 carrying *Fre1* gene) were then screened by PCR to test for presence of the three transgenes (*Fer1, Fea1* and *Fre1*). Since *Fer1* and *Fea1* are tightly linked (come from the same vector, pP0176), it is believed that if a clone is PCR positive for one of the transgenes (*Fer1* or *Fea1*), it most likely contains the second transgene [12,14]. The PCR positive *A. protothecoides* transformants carrying the *Fer1* and *Fea1* transgenes were analyzed for the presence of *Fer1* and *Fea1* transcripts by RT-PCR. Western blot experiments were carried out on some of the RT-PCR positive clones to detect *Fer1* and *Fea1* protein expression. All of the tested clones were found to contain *Fer1* and *Fea1* transcripts indicating that these transformants were transcribing the integrated transgenes. Experimental results for three strains, wild type (WT), 175/176-113 and 175/176-119 are presented. This annotation corresponds to the vectors inserted into the cells (pP0175 and pP0176) and the isolate number (113 and 119), respectively. For brevity, they will be referred to as WT, strain 113 and strain 119 in the remainder of the text.

To characterize the iron uptake and storage properties, growth media with enough chelator to make excess iron biologically available, but not enough to hinder growth, were developed (Phycal). Sueoka's Modified High Salt (MHS) media contained eight times the standard concentration of iron with the molecular equivalent of ethylenediaminetetraacetic acid (EDTA). The experiments were performed within a range of iron concentrations from one (2.06 mg/L of elemental iron) to eight (16.48 mg/L of elemental iron) times the standard concentration in MHS, where the molar equivalent of EDTA was used from 17.31 mg/L to 276.96 mg/L, respectively. The algae cultures were harvested at either the late log phase or the stationary phase, the nearly two liter (2 L) cultures were centrifuged to a pellet, then resuspended in EDTA buffer, re-centrifuged, lyophilized, and sent out for intracellular iron analysis. The elemental iron in the dry cell mass was measured by inductively coupled plasma atomic absorption (ICP–AA, National Testing Laboratories, Ltd., Cleveland, OH).

2.2. Magnetic deposition microscopy (MDM)

The magnetic algae separation conditions were tested under precisely controlled fluid flow and magnetic field settings. Magnetic Deposition Microscopy or (MDM) is based on a previous open-gradient magnetic field separator and a thin-film magnetopheresis process developed for cell analysis [8–10]. The magnet generates high magnetic gradient in order to pull weakly magnetic cells from the flowing cell suspension and deposit the cells on an optically transparent, thin sheet of a Mylar (130 μm thick) for microscopic analysis (Fig. 1). The magnetic field was generated by a neodymium permanent magnet assembly. Three sizes of component magnet blocks, neodymium–iron–boron 42 MG Oe energy product, were purchased from Applied Magnet, Plano, TX. The steel yokes and aluminum supports were machined in-house. The interpolar gap width was 1.6 mm for each of the two interpolar gap regions; the maximum magnetic field intensity measured at the midline between the two interpolar gaps in the $0y$ direction was $B_y = 0.475$ T; when interpolated to the interpolar gap region (using Amperes 3D boundary element method field modeling software from Integrated Engineering Software, Winnipeg, Manitoba) the field was in excess of 1.4 T (Fig. 1). The direction of the resulting magnetic force acting on algae cells was essentially in the plane perpendicular to the magnet surface, reducing the problem of cell trajectories to two dimensions. Five flow channels (6.3 mm wide by 15 mm long each) were created by a cutout in a 0.25 mm thick rubber spacer sandwiched between poly-l-lysine coated, 0.13 mm thick Mylar sheet (serving as a microscopy substrate slide following completion of the MDM run) and a polycarbonate manifold. The manifold was connected to each flow channel to sample inlet and outlet tubing (FEP, 0.508 × 1.59 mm inner diameter × outer diameter, Zeus Industrial Products), Fig. 1C. One mL tuberculin syringes mounted on a Harvard PhD2000 programmable syringe pump (Harvard Apparatus, South Natick, MA) provided means for cell suspensions (500 μL each) pumping through the flow channels at precisely controlled volumetric flow rate (0.026 mL/min aspirating the sample upward and 0.013 mL/min dispensing the sample downward, for a total of two passes of the majority of the cell suspension volume over the magnet's two interpolar gaps).

The channels and corresponding tubing were primed to wet the inner surface of the Mylar slide, the flow channels and the associated tubing using 0.1% Pluronic F-68 (BASF Corp.) in DDI water at a flow rate of 0.4 mL/min. Before the sample was loaded onto the MDM the concentration of the algae sample was determined using the Z2 particle counter/size analyzer (Beckman-Coulter) with counts gates by diameter in the range from 3 μ to 8 μm). The pre-sorted sample is known as the feed, the fraction collected after sorting is the eluate and the fraction captured
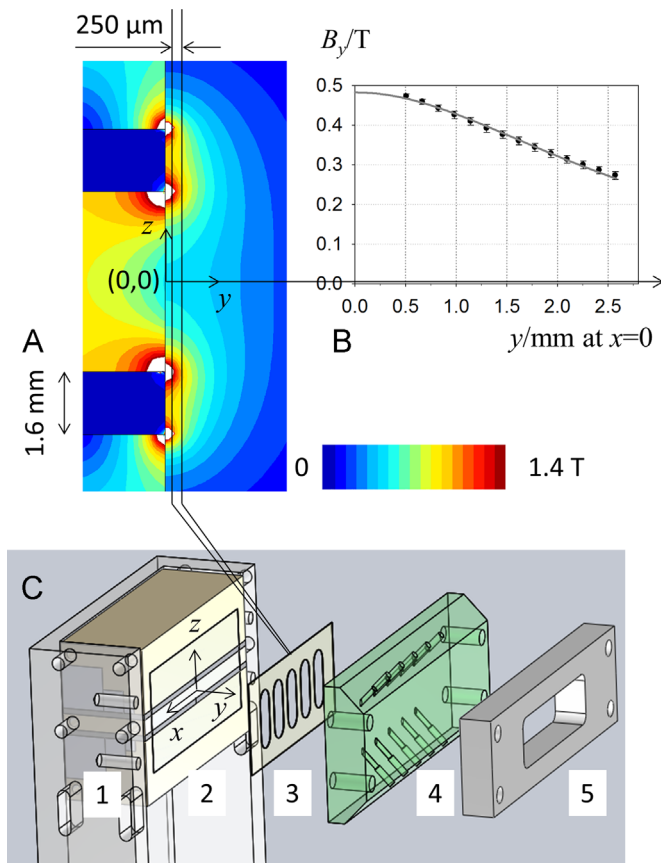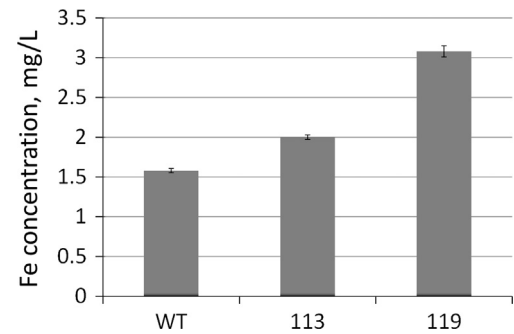
Fig. 1. (A) False color map of the magnetic field and an outline of the flow channel longitudinal section (two parallel lines) showing four "hot spots" of high magnetic field (exceeding 1.4 T) and gradient (exceeding 1000 T/m) at the edges of two interpolar gaps (blue rectangles) encountered by the algae suspension pumped up and down the flow channel in the magnetic deposition microscopy (MDM) device. The color gradation is by 0.08 T. (B) The magnitude of the magnetic field was verified by comparing the magnitude of the calculated field shown in A with the one measured along the $0y$ axis of the magnet's plane of symmetry, $x=0$. (C) Exploded view of the MDM device showing magnet assembly with two interpolar gaps (1), the Mylar slide 130 μm thick (2), the silicone rubber gasket 250 μm thick with five flow channel cutouts (3), the inlet and outlet flow manifold for connecting tubing to the sample container and the syringe pump, respectively (not shown) (4), and a platen to press all the parts together against the magnet (5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Dry mass iron concentration by ICP–AA in *A. protothecoides* cultured in MHS media modified by supplementation with $8\times$ the baseline concentration of chelated $FeCl_3$, showed increased iron incorporation in genetically modified strains 113 and 119 compared to wild type, WT. Error bars are standard deviations of at least two replicates.

Fig. 3. Percent capture by magnetic deposition of algae in MDM device depended on the chelated iron media supplementation (baseline versus $8\times$ baseline, $p < 0.0001$) and, for the high iron media only, on the type of strain (wild type, WT versus genetically modified strains 113 and 119, $p < 0.0001$). Error bars are standard deviations for sample sizes indicated by N values. There was no difference in percent capture of strains 113 and 119 at baseline iron media concentration.

on the slide is the magnetic deposit. For each channel, excess sample was loaded into 1.5 mL into which lower tubings were placed. The syringes initially contained 0.25 mL of air. The pump aspirated the sample at a flow rate of 0.026 mL/min for a total volume of 0.5 mL and stopped. Then, the sample micro-centrifuge tubes were replaced by fresh tubes to collect the eluate and the pump was set to infuse the cell suspension at 0.013 mL/min for a volume of 0.7 mL, to completely evacuate the system of fluid. The eluate algae fraction was also counted by the Coulter Counter and the difference between feed and the eluate cell number was taken as the deposited algae number on the MDM slide.
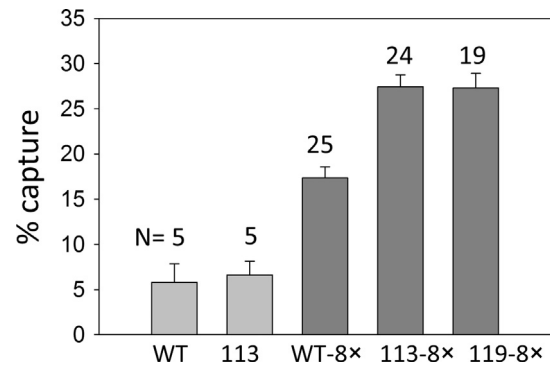
3. Results and discussion

3.1. Intracellular, elemental iron concentration by ICP–AA

The results of an experiment where the iron content was analyzed after the algae were harvested from growth in $8\times$ Fe MHS media is presented in Fig. 2. The data from this experiment

indicate that strain 119 contains the most iron as compared to WT and 113 strains.

3.2. Magnetic separation of algae due to their intrinsic magnetic susceptibility

The algae strains were processed through the MDM device to determine the flow conditions required to capture cells from the suspension. The algae that were genetically modified to uptake media iron were significantly more magnetically susceptible than the unmodified algae at $8\times$ iron media concentrations (Fig. 3). However, at the base line concentrations of media iron the genetically modified algae showed comparable deposition percentages to that of wild type algae. This suggests that the genetic modification of the algae plays a role in increasing the magnetic susceptibility of the algae but only at a high iron media concentration.

The microscopy slide of the algae deposition reveals the concentrated algae deposition at the 2 T regions of the interpolar gap (Fig. 4). A "gel scan" of the image of the slide (Image J, [19]) shows a similar profile to that calculated for magnetic particles entrained in a laminar flow inside a rectangular channel of the MDM device. The algae particles magnetophoretic mobility values used for the calculations were measured using cell tracking velocimetry (results not shown).

The results show that, in principle, the magnetic dewatering of the algae based on their intrinsic magnetization is possible. However, the high magnetic field (2 T) and gradient (approaching 1000 T/m) combined with low flow rate (up to 0.026 mL/min)
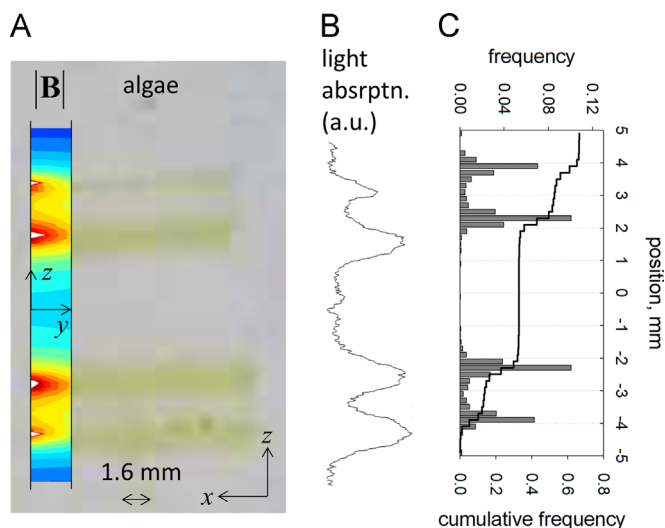
Fig. 4. Magnetic algae capture pattern on the Mylar slide (photograph in A and light absorption scan in B) fitted the location of the magnetic B field magnitude "hot spots" (inset in A) and the theoretical predictions based on calculated cell trajectory distributions in the MDM flow channel (in C).

required for the magnetic algae capture make the process prohibitively expensive on a large scale required for biofuel production. Moreover, the scaling process requires specialized high iron media composition, further increasing the cost of the operation. In comparison, the extrinsic magnetization of algae by binding of iron oxide nanoparticles remains a competitive approach to magnetically dewater algae suspensions economically on a large scale [4].

4. Conclusions

Three genes that enhance cellular iron accumulation were successfully inserted into Phycal's KRT1006. All the strains accumulate higher iron content when grown in media with higher chelated iron concentration. MDM separation results revealed that approximately 30% more of the genetically modified algae strains separated from the $8\times$ Fe culture media compared to the WT in $8\times$ Fe culture medium. There is evidence that these genetically modified stains possess enhanced magnetic properties, but not enough to make an impact on the integrated algal biofuel production system economics.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jmmm.2014.09.008.

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|B| algae light absorption (a.u.)

1.6 mm

z

x