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Breaking Dormancy: An energy-efficient means of recovering astaxanthin from microalgae†

Ramasamy Praveenkumar,† Kyubock Lee,‡ Jiye Lee and You-Kwan Oh*

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*Haematococcus pluvialis, in the dormant aplanospore (cyst) status after 30 d cultivation, accumulates high levels of a superpotent antioxidant, astaxanthin, which has been demonstrated to have enormous therapeutic benefits. However, owing to the robust structure of its trilayered cell-wall, the recovery of astaxanthin from the cyst cells remains an energy-intensive process. In the present study, a novel strategy utilizing short-period germination based on the natural life-cycle of H. pluvialis was developed as an energy-efficient pretreatment for extraction of astaxanthin using ionic liquids (ILs) as green solvents. The germination resulted in damage and deconstruction of the cyst cell-wall, and thereby facilitated the extraction of astaxanthin by ILs under room temperature. By this natural pretreatment with 1-ethyl-3-methylimidazolium ethylsulfate, for the very short reaction time of 1 min, the high astaxanthin yield of 19.5 pg/cell was obtained, which was about 82% of a conventional volatile organic solvent extraction by strong, 30,000 psi French-pressure-cell homogenization. The maximal astaxanthin-extraction yield from H. pluvialis cells was observed for 12-18 h germination. The germination rate furthermore could be improved by manipulating the nutritional composition (especially the nitrate concentration) of the culture medium. In light of these results, it can be posited that natural germination following the principles of green chemistry can be a uniquely simple method of robust microalgal cyst cell pretreatment and extraction of astaxanthin with room-temperature ILs.

Introduction

Nature has a smart strategy of protecting organisms from unfavorable environmental conditions such as nutrient deprivation, desiccation, and extreme temperatures: minimal metabolic activity, namely dormancy.1–2. Once conditions become favorable again, organisms halt their dormant status and re-initiate growth. This strategy is used by a variety of organisms, from microorganisms to plants to animals. Good examples are found with broad-leaved trees, whose metabolisms come to a standstill by loss of leaves during winter and become deciduous with spring. Haematococcus pluvialis is a representative unicellular freshwater green microalga that has such a dormant phase. This biflagellate microalga forms non-mobile resting cells, namely aplanospores (cysts), under stress conditions such as nitrogen depletion and strong insolation.3,4. During this transformation, the cells become self-protective, not only by consolidating their cell-walls, but also by accumulating high levels of secondary carotenoids such as astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione).

Astaxanthin has been attracting broad and focused attention owing to its function as a powerful antioxidant5-7. It is claimed for therapeutic applications such as immune stimulation, anti-inflammation, carpal tunnel syndrome and muscle soreness treatment8. Additionally, astaxanthin has been reported to have inhibitive effects on mammary tumors and oral carcinogens in animal models9, and is widely utilized in feed industries such as poultry and aquaculture9. Most astaxanthin, market-valued at 2,500 $/kg, currently is produced by chemical synthesis, is consumed mostly as feed additives10. For direct human consumptions, however, natural astaxanthin is preferred over the synthetic form due to a mixture of isomers and safety concerns10. H. pluvialis is considered to be an outstanding source of natural astaxanthin (~ 40,000 ppm) among other commercially used sources such as Euphausia pacifica (pacific krill, ~ 120 ppm) and Pandalus borealis (Arctic shrimp, ~ 1,200 ppm)10. Astaxanthin is highly accumulated in H. pluvialis during the development of aplanospores from vegetative cells under stress conditions. However, this is accompanied by the formation of a rigid cell-wall structure of considerable thickness (1.8 – 2.2 µm) consisting of several layers including the trilaminar sheath (TLS), secondary wall (SW), and tertiary wall (TW)11. Most notably, the outmost TLS is composed of a sporopollenin-like polymer known as algaenan, a tough and non-hydrolysable material12, 13. The cell-wall of the aplanospore, then, is remarkably resistant to physical and chemical cell disruptions, which fact complicates the astaxanthin-extraction process. Even highly energy-consuming mechanical methods such as homogenization or sonication are less efficient for cell disruption of H. pluvialis cysts and, therefrom, astaxanthin extraction, than for other microalgal species14. Harsh conditions rather, such as strong acidity with elevated temperatures or supercritical fluid, have to be applied15-17. Therefore, additional...
efforts for a scalable and also environment-friendly extraction method are required in order to achieve economic astaxanthin production from *H. pluvialis* biomass.

In recent days, being environmental-friendly solvents, ionic liquids (ILs), the salts composed of relatively large organic cations coupled with smaller inorganic or organic anions, are successfully utilized in the field of biorefinery, food and feed processing and as active pharmaceutical ingredients. ILs have been applied to microalgal biorefinery especially for cell-wall disruption and lipid extraction from *Chlorella* sp., *Chlamydomonas* sp. and *Scenedesmus* sp.. Cell lysis and lipid extraction efficiencies vary largely depending on not only the microalgal species (differing structure and cell-wall composition), but also the operating conditions, such as the IL used, dosage, and temperature applied. However, very few applications of the dormant status of microalgae, such as *H. pluvialis* aplanospores containing high concentrations of astaxanthin, have been reported. It should be also noted that conventional high-temperature IL treatments can both curtail extraction yields of temperature-labile astaxanthin and also increase operating and facility costs.

The purpose of this study was to develop an energy-efficient and environment-friendly process for extraction of high-value astaxanthin from *H. pluvialis* aplanospores (cysts) using the physiological artifice of environmental- and nutritional-condition alteration to effect conditions more favorable for dormancy breaking by room-temperature IL. This process consists of the natural germination of aplanospores, which allows them to lose their cell-wall rigidity, followed by IL-mediated astaxanthin extraction from the germinating cells. More specifically, during germination, the rigid TLS and SW of the aplanospores break open, leaving dividing cysts and zooids with weak cell-walls that are highly susceptible to chemical and/or physical treatments. More importantly still, we showed that the dividing cysts and zooid cells could still retain their astaxanthin content for a short period before becoming completely acclimatized to the favorable environment (at which point the post-division cells lose astaxanthin to synthesize other photosynthetic pigments for photosynthetic growth).

**Materials and methods**

**Microalgae and culture medium**

*Haematococcus pluvialis* NIES-144, as obtained from the National Institute for Environmental studies (NIES), University of Tokyo, Japan, was used in this study. The life-cycle of *H. pluvialis* is divided mainly into 3 stages: 1) vegetative cell growth, 2) encystment along with astaxanthin induction (vegetative cells to immature to mature cysts), and 3) germination (mature cysts to vegetative cells). NIES-C medium was used for both vegetative cell growth and cyst germination, in the following composition (per liter): 0.15 g Ca(NO₃)₂, 0.10 g KNO₃, 0.05 g β-glycerophosphoric acid disodium salt pentahydrate, 0.04 g MgSO₄·7H₂O, 0.50 g Tris-aminomethane, 0.01 mg thiamine, 3.00 ml PIV metal solution, 0.10 µg biotin, and 0.10 µg vitamin B₁₂. One liter of PIV metal solution consists of 1.0 g Na₂EDTA, 0.196 g FeCl₃·6H₂O, 36.0 mg MnCl₂·4H₂O, 22.0 mg ZnSO₄·7H₂O, 4.0 mg CoCl₂·6H₂O, and 2.5 g Na₂MoO₄·2H₂O. For astaxanthin induction, nitrogen (N)-free medium (NIES-N) was prepared by substituting (per liter) 0.13 g CaCl₂·2H₂O and 0.07 g KCl for Ca(NO₃)₂ and KNO₃ from the NIES-C medium, respectively. The media were sterilized by filtration through a 0.20 µm membrane (Sartorius Stedium Biotech., Germany), and the pH was adjusted to 7.5.

**Vegetative cultivation of *H. pluvialis***

One colony of *H. pluvialis* grown on an agar-plate was transferred to a 250 mL Erlenmeyer flask (working volume, 100 mL; NIES-C medium). The flask was incubated for 14 d in a shaking incubator (IS-971RF, Lab Companion, Korea) at 25°C and 150 rpm, and thereafter was used as the inoculum for the main photobioreactor culture. Light was continuously supplied at 40 µmol/m²·s by white-fluorescent lamps.

A vegetative cell culture was performed in a Pyrex-glass bubble-column photobioreactor (b-PBR) (length, 35 cm; inner diameter, 3.7 cm; working volume, 500 mL) with the NIES-C medium. The inoculum concentration was adjusted to an initial optical density (OD) of 0.1 at 680 nm. The b-PBR was continuously supplied with 5% (v/v) CO₂ in air at 0.4vvm from the bottom of the reactor, as previously optimized (data not shown). The supplied gas was passed through a 0.2 µm PTFE venting filter (Minisart 2000, Sartorius Stedium Biotech., Germany) and controlled by mass flow controllers (MK Precision, Korea) and flow meters (Dwyer Instruments Inc., USA). The b-PBR culture was incubated for 15 d in a plant-growth chamber (GC-300, JEIO TECH, Korea) at 25°C and illuminated with 25 µmol/m²·s (light/dark 12:12 h cycles) by white-fluorescent lamps.

**Encystment and astaxanthin induction of *H. pluvialis***

The astaxanthin induction of the vegetative *H. pluvialis* cells was performed using a conical-shape (funnel-like) PBR (f-PBR) (working volume, 300 mL). The vegetative cells (OD ~ 0.8 at 680 nm) from the b-PBR were harvested by centrifugation at 3,000 rpm for 2 min, washed with fresh NIES-N medium, and then transferred to the original or a modified NIES-N medium. The f-PBR cultures were incubated for 15 d in another plant-growth chamber at 25°C. The aeration condition was the same as for the b-PBR culture. Light was continuously supplied at 69 µmol/m²·s by white-fluorescent lamps.

**Germination of *H. pluvialis* cysts**

The influence of the nutritional composition of the NIES-C medium on the germination efficiency of *H. pluvialis* cysts was tested as indicated in Table 1. The mature red cysts from the f-PBR were collected by centrifugation at 3,000 rpm for 2 min, washed with NIES-C medium, and transferred to the original or a modified NIES-C medium. The cells were incubated in a 250 mL Erlenmeyer flask (working volume, 100 mL) in a shaking incubator (IS-971RF, Lab Companion, Korea) at 25°C and 150 rpm. Light was continuously supplied at 40 µmol/m²·s for 24 h.
**Astaxanthin extraction from germinating cells using ILs**

Three classes of ILs, namely 1-ethyl-3-methylimidazolium (Emim), 1-butyl-3-methylimidazolium (Bmim) and 1-butyl-3-methylpyridinium (Bmpy), were tested for their efficiencies for astaxanthin extraction from germinating cysts. The ILs and their structures are listed in Table 2. Briefly, 1 mL of culture aliquot was collected before and after 12 h germination in 2 mL micro centrifuge tubes by centrifugation (3,000 rpm, 2 min) and washed with distilled water. Astaxanthin from cell pellets was extracted with 0.5 mL IL by 1 min vortexing under room temperature (28°C). The IL solution was then separated by additional centrifugation. For complete astaxanthin extraction, the remaining cell paste was again treated by 0.5 mL of the same IL and incubated for 24 h under the dark condition. For the recovery and further quantitative analysis of the free astaxanthin, the pigment from 250 µL IL was extracted with 1 mL of ethyl acetate via a simple liquid-liquid extraction process.

**Astaxanthin quantification**

The time-course change of the astaxanthin content of *H. pluvialis* cells during the germination process was investigated. The culture broth was collected at 6 h intervals and homogenized using a French-pressure-cell (Thermo Electron Corp., USA) at 30,000 psi, as previously optimized (data not shown). For astaxanthin extraction, 1 mL of the homogenate was mixed vigorously with 5 mL of ethyl acetate for 10 min under room temperature. The mixture was separated by centrifugation at 4,000 rpm for 10 min. The astaxanthin-containing ethyl acetate solution was collected and evaporated using a rotary vacuum evaporator (EZ2 PLUS, Genevac Ltd., UK). The astaxanthin extract was mixed with 1 mL of 0.025 N NaOH in freshly prepared methanol and then saponified at 4°C for 70 min under previously optimized conditions (data not shown).

The free (de-esterified) astaxanthin content was analyzed using a high-performance liquid chromatograph (HPLC) (1260 series, Agilent Technologies, USA) equipped with a variable-wavelength detector (VWD) and a YMC Carotenoid column (C30, 5 µm, 250 × 4.6 mm; YMC Co., Japan). Liquid sample was purified through the column. The mobile phase consisted of solvent A (methanol/methyl tertiary butyl ether (MTBE)/1% (w/v) phosphoric acid, 81:15:4, v/v) and solvent B (methanol/MTBE/1% (w/v) phosphoric acid, 16:80:4, v/v). For simultaneous separation of free astaxanthin and fatty-acid-containing astaxanthin esters, the following gradient procedure was used: 0% of solvent B for 15 min; a linear gradient from 0% to 100 of solvent B for 27 min; 0% of solvent B for 35 min. The flow rate was 1.0 mL/min. The peaks were measured at a wavelength of 474 nm to facilitate the astaxanthin detection in comparison with the chemical standard (Dr. Ehrenstorfer GmbH, Augsburg, Germany) (see Figs. S1 and S2 for characterization information). The column temperature and injection volume were maintained at 30°C and 20 µL, respectively.

**Table 2** List of ILs used for astaxanthin extraction from germinating *H. pluvialis* cysts

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Structure</th>
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<tr>
<td>1-ethyl-3-methylimidazolium acetate</td>
<td>[Emim] OAc</td>
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<tr>
<td>1-ethyl-3-methylimidazolium tetrafluoroborate</td>
<td>[Emim] BF₄⁻</td>
<td></td>
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<tr>
<td>1-ethyl-3-methylimidazolium ethylsulfate</td>
<td>[Emim] EtSO₃⁻</td>
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<td>1-ethyl-3-methylimidazolium methysulfate</td>
<td>[Emim] MeSO₃⁻</td>
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<tr>
<td>1-butyl-3-methylimidazolium methysulfate</td>
<td>[Bmim] MeSO₃⁻</td>
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<tr>
<td>1-butyl-3-methylpyridinium methysulfate</td>
<td>[Bmpy] MeSO₃⁻</td>
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**Other analytical methods**

The OD was measured at 660 nm by UV-VIS spectrophotometry (Optizen 2120UV, Mecasys Co., Korea). The pH and light intensity were determined using a pH meter (DKK-TOA Co., Japan) and a quantum meter (LI-250A, LI-COR Inc., USA), respectively. The cells in the germination stage were classified into 4 types including mature, dividing, releasing, and empty, and were counted at 6 h intervals using an improved Neubauer counting chamber (C-Chip, DHC-N01, iNCTYO, Korea). Germination of *H. pluvialis* cells and astaxanthin extraction from them were observed by bright-field and fluorescence microscopy (Axio Imager.A2, Carl Zeiss, Germany). Blue signals (for IL uptake into cells) and chlorophyll auto-fluorescence were detected with a filter set 49 (Excitation filter: 365 nm; BS FT: 395 nm; EM LP: 445-450 nm) and a long pass filter set 09 (Excitation filter: 450-490 nm band pass; BS FT: 510 nm; EM LP: 515 nm), respectively. The images were captured with an AxioCam HRC CCD camera equipped with AxioVision software (Carl Zeiss, Germany).
pretreatment. When a mature cyst of \( H. \) pluvialis at temperature condition before and after 12 h-germination, it appeared intact, showing no effect when mixed with [Bmim] MeSO\(_4\). As the germination progressed, the cyst cell underwent significant changes both morphologically and physiologically. The increase of the cell size due to cell divisions (Figs. 1b and 1f) resulted in cracking of the TLS and SW, leaving the dividing cyst covered only by a weak, flexible TW (Figs. 1c and 1g). With the development of zooids, the TW also broke open, releasing motile zooids cells into the medium (Figs. 1d and 1h). A 24 h-germination movie is available as Supplementary Information Video S1. It should be noted that the overall red-color intensity, as an indirect indicator of astaxanthin, remained, albeit of sequential transformations from a mother cyst to a dividing cyst to motile zooids, implying a new biotechnological tool for astaxanthin extraction.

**Results and discussion**

**Germination of \( H. \) pluvialis cyst**

Fig. 1 shows time-course profiles of the microscopic differentiation and a schematic illustration of a mature red cyst of \( H. \) pluvialis during germination in NIES-C medium. The thick, trilayered cell-wall structure including the TLS, SW, and TW was observed in a mature cyst cell after 30 d incubation (Figs. 1a and 1e)\(^1\). As the germination progressed, the cyst cell underwent significant changes both morphologically and physiologically. The increase of the cell size due to cell divisions (Figs. 1b and 1f) resulted in cracking of the TLS and SW, leaving the dividing cyst covered only by a weak, flexible TW (Figs. 1c and 1g). With the development of zooids, the TW also broke open, releasing motile zooids cells into the medium (Figs. 1d and 1h). A 24 h-germination movie is available as Supplementary Information Video S1. It should be noted that the overall red-color intensity, as an indirect indicator of astaxanthin, remained, albeit of sequential transformations from a mother cyst to a dividing cyst to motile zooids, implying a new biotechnological tool for astaxanthin extraction.

**Model of IL-based astaxanthin extraction from germinating cells**

Fig. 2 illustrates the microscopic changes involved in IL-based astaxanthin extraction from \( H. \) pluvialis cells under the room temperature condition before and after 12 h-germination pretreatment. When a mature cyst of \( H. \) pluvialis was initially mixed with [Bmim] MeSO\(_4\), it appeared intact, showing no effect of the IL (Fig. 2a). After 12 h-germination by contrast, and to our surprise, the spontaneous extraction of red-colored-astaxanthin-containing lipid droplets from the dividing cyst and released zooids was observed within 2 min, as indicated by the black arrows in Figs. 2e and 2i. Video S2 is a real-time movie of the extraction of astaxanthin in the form of lipid droplets after 5 min [Bmim] MeSO\(_4\) treatment. Using the property of [Bmim] MeSO\(_4\) according to which it fluoresces at around 450 nm, its uptake by different cell types was monitored microscopically. The mature red cyst did not uptake [Bmim] MeSO\(_4\), and so its presence (blue color) inside the cell was not observed (Fig. 2b); instead, [Bmim] MeSO\(_4\) bound to the cyst’s outer layer, showing a strong blue fluorescence. The dividing cyst and the released zooids, contrastingly, could uptake [Bmim] MeSO\(_4\) to emit blue-color fluorescence (Figs. 2f and 2j). The replicated images of blue fluorescence by [Bmim] MeSO\(_4\) and of red auto-fluorescence originating from chlorophyll clearly showed the absorption degrees of [Bmim] MeSO\(_4\) according to the cell types: none for the mature cyst (Figs. 2b and 2c); partial for the dividing cyst (Figs. 2f and 2g); complete for the released zooids (Figs. 2j and 2k). As illustrated in Figs. 1f and 1g, the dividing cyst lost the TLS along with the SW. Hence, it is plausible that in the mature cyst, the toughest layers of the cell-wall, namely the TLS and SW, prevented [Bmim] MeSO\(_4\) penetration into the cell (Figs. 2a and 2d), whereas, in the dividing cyst, the presence of only a weak TW allowed it (Figs. 2e and 2h). Furthermore, the motile zooids, with only a delicate layer of plasmalemma covering them, were highly susceptible to [Bmim] MeSO\(_4\), thus readily allowing complete penetration (Figs. 2i and 2l; Table S1).

In the case of the mature cyst, the cell was intact at elevated temperature (60ºC, 20 min) in the presence of [Bmim] MeSO\(_4\) (Fig. S3). Moreover, no damage to the cell-wall was evident, even up to 90ºC until 90 min (data not shown). The cell-wall of a mature red cyst contains a TLS made up of an acetolysis-resistant aliphatic biopolymer known as algaenan, which puts up great resistance to physical and chemical treatments. This protective function of the...
Fig. 2 Microscopic images and schematic illustrations of IL-based astaxanthin extraction from *H. pluvialis* cells before and after germination. (a - d), Initial mature cyst. (e - h), Dividing cyst. (i - l), Released zooids. DIC (a, e, i) mode shows extraction of lipid droplets containing red-colored astaxanthin from germinating cells (see black arrows). Blue color fluorescence (b, f, j) under filter 49 represents uptake of IL into cell. Red color fluorescence (c, g, k) under filter 09 represents chlorophyll auto-fluorescence. Scale bars represent 20 µm. The schematic diagrams (d, h, l) represent the proposed models of IL uptake and astaxanthin extraction. (d), Mature cyst impermeable to IL; astaxanthin extraction occurs efficiently. Abbreviations: AXT, astaxanthin; CW, cell-wall; IL, ionic liquid; PW, primary wall; SW, secondary wall; TLS, trilaminar sheath; TW, tertiary wall; After 12 h germination of mature cyst (a, b, c) in NIES-C medium, the dividing cyst (e, f, g) and zooids (i, j, k) were collected for IL treatment. One hundred (100) µL culture (2.3 × 10⁵ cells/mL) was harvested, washed with distilled water and extracted with 50 µL of [Bmim] MeSO₄ for 2 min

<table>
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<tr>
<th>Optical micrograph</th>
<th>Fluorescence micrograph</th>
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<tr>
<td>Mature cyst</td>
<td>IL</td>
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<tr>
<td>Dividing cyst</td>
<td>Chlorophyll</td>
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<td>Released zooids</td>
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Astaxanthin-extraction efficiencies of various ILs after germination pretreatment

Imidazolium- and pyridinium-based ILs with different anions were tested for their efficiencies in extracting astaxanthin from germinating cysts of *H. pluvialis* (Fig. 3, Table 2). Here, a natural pretreatment method for cell-wall damage through germination of mature cysts was compared with the strongest, most energy-intensive pretreatment method that of French-pressue-cell homogenization at 30,000 psi previously optimized (data not shown). The optimal germination time in NIES-C medium was determined to be 12 h (see below). Similarly, the efficiencies of the nonvolatile, greener-solvent ILs were compared with the volatile ethyl acetate conventionally used for astaxanthin extraction. When the 12 h-germinated sample was extracted with [Emim] EtSO₄ over a very short reaction time of 1 min at room temperature, the high astaxanthin yield of 19.5 pg/cell was obtained, which corresponded to about 82% of the yield obtained by the conventional, energy-intensive high-pressure homogenization-based ethyl acetate extraction method (Fig. 3). Significantly, this indicates that the combination of germination pretreatment and IL extraction has a high potential as a new, powerful technique of astaxanthin extraction from *H. pluvialis* cysts that does not require excess energy for high temperature and/or intensive mechanical cell-disruption, and which also avoids or minimizes the use of volatile organic solvents. Previous researches with *H. pluvialis*, with varying culture conditions, reported astaxanthin extraction yields of 20-30 pg/cell with 90% acetone and 48 pg/cell with acetone:methanol (1:2; v/v) with different pretreatment methods. It should be noted that the astaxanthin yield not only depends on the extraction method but also on the cultivation conditions and type of stress applied for astaxanthin induction. What we have shown here is that we were
able to get high extraction yield of astaxanthin through a greener process by using ILs along with a process of germination within a very short time of 1 min without spending energy. Furthermore, after additional 24 h incubation at room temperature (Fig. S4), the maximal astaxanthin yield, 32.5 pg/cell, was obtained, which was 1.4-fold higher than that achievable with the conventional extraction method. Beyond such efficiency, ILs also have high synthetic flexibility, which allows us to design a solvent for a specific reaction\[13\]. In this way, these “designer solvents” have high selectivity for the target compounds which is unlikely with the volatile organic solvents\[10\].

The extraction efficiencies of the tested ILs were in the order [Emim] EtSO\(_4\) > [Bmim] MeSO\(_4\) > [Emim] MeSO\(_4\) > [Bmpy] MeSO\(_4\) > [Emim] BF\(_4\) > [Emim] OAc (Fig. 3). With allyl sulfates (MeSO\(_4\) and EtSO\(_4\)) as anions, the imidazolium-based ILs ([Bmim] and [Emim]) were more efficient than the pyridinium-based IL ([Bmpy]). Among the 1-ethyl-3-methylimidazolium ([Emim]) ILs tested, anion groups such as BF\(_4\) and acetate showed lower astaxanthin-extraction efficiencies than the allyl sulfates such as MeSO\(_4\) and EtSO\(_4\). These results indicate that dividing cysts and zooids of *H. pluvialis*, notwithstanding their weaker cell-walls, are susceptible only to a few, not all ILs; this implies, in turn, that the permeability of ILs into cells is largely affected by the structural and chemical properties of cations and anions.

Fig. 3 Astaxanthin extraction yield from germinating *H. pluvialis* cells using various ILs against ethyl acetate (EA). For IL-based extraction, after 12 h germination of mature cysts, a 1 mL sample was collected and treated with 0.5 mL. ILs for 1 min at room temperature. In the case of EA, 20 mL of initial mature cysts were homogenized using the French-pressure-cell homogenization at 30,000 psi. One (1) mL of the homogenate was extracted with 5 mL of EA for 10 min. Astaxanthin was quantified as pg/cell based on the absolute cell count value.

Sen et al.\[33\] comparing the toxicities of imidazolium-based and pyridinium-based ILs to *Chlamydomonas reinhardtii* based on cell growth, reported significant inhibition effects by the former (due to penetration into cells) but only negligible effects by the latter (due to the glycoprotein-rich cell-wall). They proposed that the higher cell permeability of imidazolium-based ILs is related to the physiological and biochemical characteristics of the imidazole base, which serves as the side chain of the amino acid histidine readily taken up by cells\[33\]. Increases in the alkyl chain (lipophilicity) of the cations in ILs reportedly enhances the permeability of ILs into *Scenedesmus quadricauda* and *C. reinhardtii* cells\[36\]. This is in agreement with the higher astaxanthin-extraction yield of [Bmim] MeSO\(_4\) relative to [Emim] MeSO\(_4\) in the present study. However, on the basis of the maximum extraction efficiency obtained with [Emim] EtSO\(_4\), it could be supposed that the alkyl chain-length of the anions carries more significance than the chain-length of the cations in terms of *H. pluvialis* cell permeability. [Emim] BF\(_4\) is widely considered to be permeative with animal \[37\] and plant cells \[38\] but to be ineffective for astaxanthin extraction from germinating *H. pluvialis* cells. This clearly shows that microalgae with diverse cell-wall properties respond differently to specific ILs. In fact, the elucidation of such differential cell-wall behavior towards ILs would be an interesting focus of future research.

Following extraction, the astaxanthin from ILs were readily recovered through a simple biphasic separation with ethyl acetate (Fig. S5). This process not only allows the recovery of astaxanthin but also the ILs for subsequent reuse. ILs have been considered to be especially attractive green solvents, due to properties such as good thermal stability, low vapor pressure, and high boiling point, that makes their recovery and recycling easy\[39\]. In this study, the recovered astaxanthin extract, generally the astaxanthin esters, were purified through saponification to form free form of trans-astaxanthin (Fig. S2). For scale-up processes, high pure form of trans-astaxanthin from saponified extracts could be obtained through techniques such as high-speed-counter-current chromatography\[39\], purification using IL-based monolithic cartridges\[40\] and molecularly imprinted polymer cartridges\[41\]. Moreover, recycling of ILs also gain equal importance, where the impurities in the recovered raw ILs could be removed by passing through a charcoal column followed by evaporation under reduced pressure and overnight drying\[41\].

**Control of germination rate by nutritional manipulation**

Fig. 4 shows the morphological changes undergone by *H. pluvialis* cysts over the course of 24 h germination in the original NIES-C medium. The cells were classified under light microscopy into the following 4 groups: mature, dividing, releasing, and empty. After 12 h of incubation, ~58% of the cysts were germinated, constituting dividing cells, released cysts and empty cyst walls, which latter indicated that the zooids had already been released into the medium. By the end of 24 h of incubation, almost 83% of cysts were germinated, and about 50% of the cells had already released their zooids. Interestingly, the astaxanthin content compared with that of the initial mature red cysts had increased by ~20% after 12 h germination when measured by the high-pressure homogenization-assisted ethyl acetate extraction method (Figs. S6 and S7). The carotenogenic genes could be transiently induced when the *H. pluvialis* cysts were introduced into the nutrient-rich growth medium \[42\]. Thus it is conceivable that the increase in the astaxanthin yield during 12 to 18 h of germination might have been due not only to the structural changes in the cell-walls resulting in improved astaxanthin extraction, but also to the momentary induction of this secondary carotenoid. Nevertheless, the increase in the astaxanthin yield through the germination process is an added advantage to its usefulness as a natural pretreatment for extraction. After 12 h of germination, the astaxanthin content in the cells decreased, and finally, at 24 h, was reduced by ~30%
(Fig. S6). The decrease of astaxanthin content in *H. pluvialis* is thought to be due to active degradation of this secondary carotenoid into accumulated primary carotenoids and photosynthetic pigments such as chlorophylls for photosynthetic cell growth.²⁰,⁴¹

From the perspective of the extraction-process economics, it is highly desirable to improve the germination rate of *H. pluvialis* cyst cells. To that end, the effect of nutritional condition was investigated according to various compositions of NIES-C medium (Table 1). In the results, the germination rate significantly varied with the media used (Fig. 4 and Table 1). Neither the 2 × (vitamins alone) nor the 2 × (all components) condition in the NIES-C medium significantly changed the germination rates. By contrast, the NIES-C medium with either the 2 × nitrate or 2 × (nitrate and vitamins) condition accelerated the germination of *H. pluvialis* cysts. Specifically, under the 2 × nitrate condition, after 12 h germination, about 81% of the cysts had germinated, among which about 60% were in the dividing stage.

![Relative distribution](image)

**Fig. 4** Effect of nutritional composition of NIES-C media on 24 h germination of *H. pluvialis* cysts. Original (1x) and modified NIES-C with 2 × all components (2x), 2 × vitamins (2x vit), 2 × nitrate (2x NO₃⁻), and 2 × (vitamins and nitrate) (2x vit+NO₃⁻) were used as germination media. Cells were classified into 4 types including mature, dividing, releasing, and empty, and counted using an improved Neubauer counting chamber. Mature red cysts (~2 × 10⁶ cells/mL) were transferred to 100 mL of fresh media and incubated in a shaking incubator at 25°C and 150 rpm. The light was continuously supplied at 40 µmol/m²s.

It should be noted that the dividing cysts still retained as much astaxanthin content as the initial cysts, and therefore could be utilized to achieve higher astaxanthin yields. These results demonstrate that the germination rate of *H. pluvialis* cysts can be improved by nutritional manipulation, and also that the N-source (nitrate) is a key nutrient favoring the process of cyst germination as a pretreatment for astaxanthin extraction. Previous studies have shown that the germination of aplanospores is relatively higher with ammonium carbonate when compared with potassium or sodium nitrate as a nitrogen source.⁴² Moreover, alternating supply of light and dark cycles (16:8 h) and cyclic exposure of aplanospores to low and high temperature (0 °C for 5 min; 30 °C for 10 min; 3 cycles) were shown to improve the process of germination.²⁰ Further improvements of key components of the germination process would be a worthy subject for further investigation.

**Conclusions**

Here we conceptualized an energy-efficient biotechnological *H. pluvialis* hard-cyst pretreatment alternative that utilizes the process of germination to facilitate extraction of astaxanthin using ILs as green solvents. A simple “breaking dormancy” strategy resulted in loosening of the hard cyst walls of *H. pluvialis*, which allowed the ILs to penetrate the cells and to successfully extract the astaxanthin-containing lipid droplets. Of the several ILs tested, [Emim] EtSO₄ efficiently extracted 19.5 pg/cell astaxanthin from the germinating cysts over the very short reaction time of 1 min under ambient conditions. Moreover, we showed that a controlled germination of cysts for around 12-18 h resulted in 1.2-fold-increased astaxanthin yield compared with the initial mature cysts.

The germination rate also could be improved through nutritional manipulation of the germination medium. Although the present study is not directly leading to a practical application, the demonstration of exploiting mature cysts germination process as a promising greener pretreatment strategy could facilitate the efficient extraction of astaxanthin from *H. pluvialis* biomass while cutting a significant portion of downstream costs.

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**Notes**

⁵⁻ Biomass and Waste Energy Laboratory, Korea Institute of Energy Research (KIER), Daejeon 305-343, Republic of Korea. Fax: +82 42 860 3495; Tel: +82 42 860 3597; E-mail: skoh@kier.re.kr (Y.-K. Oh)

† Electronic Supplementary Information (ESI) available: UV-vis and HPLC characterization of astaxanthin extracts, microphotographs of mature red cyst treated with IL, recovery process of astaxanthin form IL, time course changes in the relative astaxanthin yield during the process of germination of mature cyst and time lapse videos of germination of mature red cysts and IL-based astaxanthin extraction from the dividing cyst. See DOI: 10.1039/b000000x/

‡ These authors contributed equally to this work

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A highly energy-efficient natural pretreatment process of germination to assist ionic liquid-based extraction of astaxanthin from Haematococcus pluvialis is developed.