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Nitrogen Assimilation of Hydrocarbon Producing Algae, *Botryococcus braunii* UTEX-572

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The effect of nitrate,nitrite and ammonia as inorganic nitrogen sources on the modulation of nitrogen metabolism of *Botryococcus braunii* UTEX-572 has been studied under aeration. The primary process in the regulation of nitrogen metabolism by this alga has the nitrate uptake system. This uptake of nitrate operation was immediately inhibited by the presence of 0.5 mM of ammonium and reversed by 0.2~0.3 mM ammonium. When cell were exposed to 5 mM of ammonium for 24 hours the activity of nitrate reductase became inactive.

Interest in *Botryscoccus braunii* has historically focused upon its role in the formation of oil deposits (2, 3). It has been suggested that *Botryscoccus braunii* might be a renewable source of liquid hydrocarbons, largely because it forms massive floating blooms that sugget the potential for large scale cultivation and efficient harvesting (12). However, this potential has not been adequately investigated because *B. braunii* has a slow growth rate and low hydrocarbon productivity. We cultured *B. braunii* in a medium containing nitrate as a nitrogen source. Though ammonium used by the algae as a precursor of amino acids, after cultivation the algae was killed in a medium containing ammonium as the sole nitrogen source. This death of *B. braunii* may be explained by its slow growth and low hydrocarbon productivity in the laboratory-scale cultivation. We report some observations on the effects of nitrate, nitrite and ammonium as nitrogen sources on modulation of the inorganic nitrogen metabolism of *B. braunii* UTEX-572 under mild aeration.

MATERIALS AND METHODS

Algal Strain

Botryococcus braunii UTEX-572 was obtained from the Austin Algal Culture Collection (University of Texas at Austin).

Culture Conditions

Maintenance cultures were grown aseptically at 25℃ in 2 *l* culture vessels fitted with sintered glass bubblers. Cultures were continuously aerated with ambient air, or 1% CO_2 enriched air, at a rate of 0.5 vvm. Illumination was provided by cool-white fluorescence tubes alternated with glow lux tubes on a 12:12 cycle. The light intensity at the surface of the culture was 14,000 ergs/sec/cm^2. Each vessel containing 1,500 m*l* of fresh medium was inoculated with 100 m*l* of 1-week-old preculture. The standard defined medium (Chu-13) contained the following components (mg/*l*): KNO_3; 100, K_2HPO_4; 20, $MgSO_4 \cdot 7H_2O$; 50, $CaCl_2 \cdot 2H_2O$; 20, Fe-citrate; 5 and citric acid; 50 (5).

Cell Preparation for Nitrogen Assimilation

Cells were harvested after 2 days growth when nitrate reductase activity was high. Cell suspensions were centrifugated at 3,000 g for 5 minutes, then cells were washed twice with a nitrogen free medium and resuspended in a graduated culture medium. In order to standardize experimental conditions cell harvesting was done in dark conditions and cell concentrations were adjusted to $Abs_{560\ nm}=0.4$ (0.7 g/*l*).

Effect of Ammonium and Nitrite on Nitrate Uptake

Nitrate uptake was assayed at different nitrite concent-

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ration. Measurements were made in the presence of 0.0, 0.3, 0.4, 0.6, 0.8 mM of ammonium or in the presence of 0.0, 0.5 mM of nitrite. Nitrate and nitrite were added as potassium salts and ammonium was added as chloride.

Effect of pH on Nitrate Use

Nitrate uptake and nitrite release were assayed at pH values of 6.0, 7.0, 8.0, 9.0 and 10.0. Based on a Chu-13 medium, culture media were prepared at values of 6.0, 7.0, and 8.0, which were adjusted with 0.02 M of phoste buffer, or pH 9.0, and 10.0 which were adjusted with 0.02 M of carbonated buffer. Nitrate was 5 mM and the cell used in this experiment were treated with nitrogen starvation for 6 hours.

Crude Extract Preparation (6)

After 2 days growth cells were harvested by centrifugation at 3,000 g for 5 minutes, washed with a 50 mM of MOPS(morpholinopropane sulfonic acid) buffer at pH 7.0, and frozen at −20℃ for 24 hours. Frozen cell were thawed in a MOPS buffer (0.2 g of wet cells/ml). The suspension was slowly stirred for 20 minutes at 0℃, then centrifugated at 27,000 g for 10 minutes. The supernatant was concentrated by ultra membrane filteration and used as a crude extract.

Activity of Nitrate Reductase *in vitro* (8)

The *in vitro* assay of active BVH-nitrate reductase was performed using 1 ml of crude extract dissolved in buffer solution at pH 4.0, 5.0 (0.05 M citrate buffer), pH 6.0, 7.0, 8.0 (0.05 M phosphate buffer), and pH 9.0 and 10.0 (0.05 M carbonate buffer).

In addition, 0.9 ml of a mixture of 0.1 mM of KNO_3 and 0.2 μM of BV (benzyl viologen, Sigma) dissolved in the same buffer solution mentioned above, to each assay sample was added. The reaction was started by the addition of 1 mg of $Na_2S_2O_4$.

After 10 minutes of incubation at 30℃ the reaction was stopped by vigorous shaking until oxidation of dithionate was completed. The amount of nitrite formed was then determined.

Analytical Methods

Nitrate, nitrite and ammonium were measured after removal of the cells by filtration. The amount of nitrate was monitored by measuring optical absorbance at 210 nm (4), nitrite was measured by the Griess-Ilosvay color reaction (9) and the amount of ammonium was estimated by Nesslerization (7).

RESULTS AND DISCUSSION

Nitrate Assimilation

When nitrate was present in the culture medium as the sole nitrogen source at the beginning of the light exposure period, *B. braunii* excreted nitrite and ammonium steadily up to 0.2~0.3 mM as a result of nitrate consumption (Fig. 1). Nitrate can enter the cell simultaneously by carrier-mediated transfer and diffusion processes (10). Once inside the cell part of the nitrate is reduced to nitrite. Nitrite reductase activity makes possible the reduction of some nitrite to ammonium, which can be incorporated into amino acids by a number of assimilatory pathways.

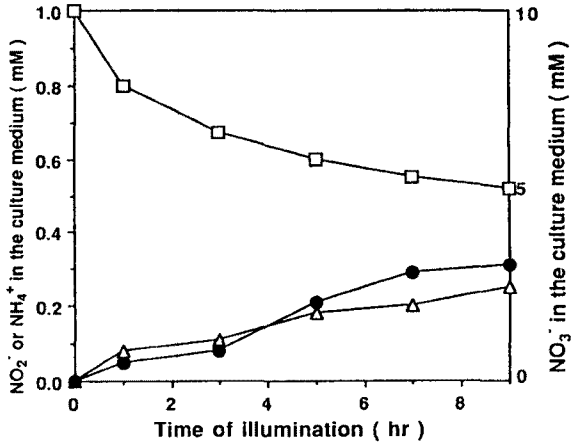

Fig. 1. Rates of NO_3^-, uptake, and NO_2^- and NH_4^+ release of *B. braunii* during illumination.
NO_3^+ (□), NO_2^- (△) and NH_4^+ (●)

Effect of Nitrite and Ammonium Ion on Nitrate Assimilation

Fig. 2(a) shows that, if in addition to nitrate 0.5 mM of nitrite is present in the medium before light exposure, the cell releases ammonium exclusively associated with nitrate consumption. However, when the ammonium amount in the medium is above 0.5 mM, reduction of nitrate stops. As shown in Fig. 2(b), when 0.8 mM of ammonium and 0.5 mM of nitrite are present with 5 mM of nitrate, the cells preferentially use ammonium until its amount is reduced to 0.2 to 0.3 mM. Then reduction of nitrate starts while amount of nitrite remains unchanged.

This suggests that the rate of nitrate and nitrite assimilation is almost the same.

That is to say, nitrite reductase may be engaged with nitrate reductase in this algal system. When 0.8 mM of ammonium, together with 5 mM of nitrate, were present in the medium (Fig. 2(c)) prior to light exposure, only the conentration of ammonium was assimilated to completion, while reduced to nitrate started to be reduced when the amount of ammonium was 0.2~0.3 mM. This result confirmed that the reduction of nitrite is coupled with that of nitrate and is not inhibited by nitrate.

It is known that ammonium indirectly affects nitrate uptake by inhibiting nitrate reductase or directly influenc-

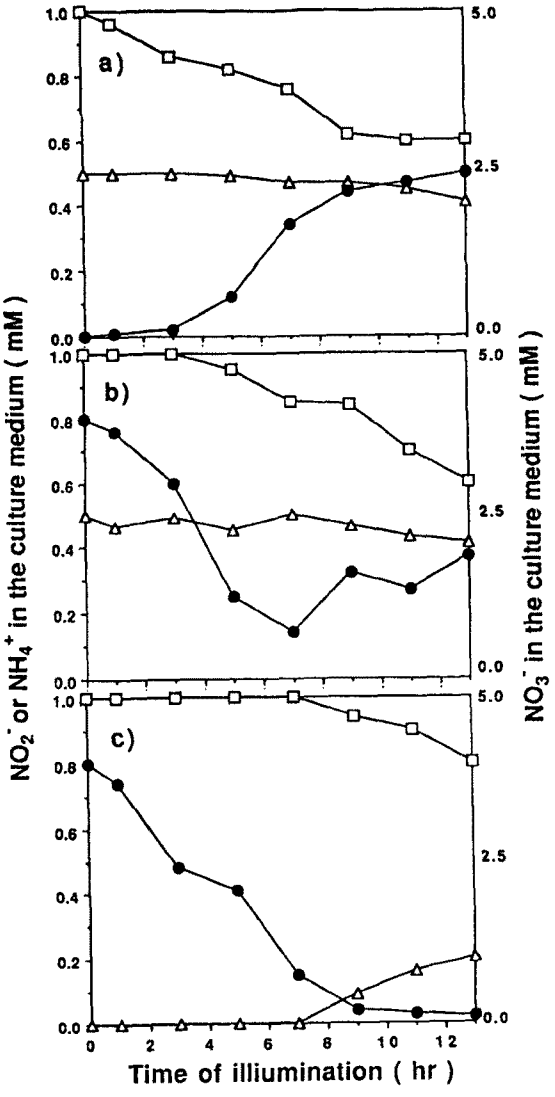

Fig. 2. Effect of both NO_2^- and NH_4^+ on NO_3^- utilization NO_3^- (□), NO_2^- (△) and NH_4^+ (●).
Cells were dark-treated before the illumination (see materials and methods).

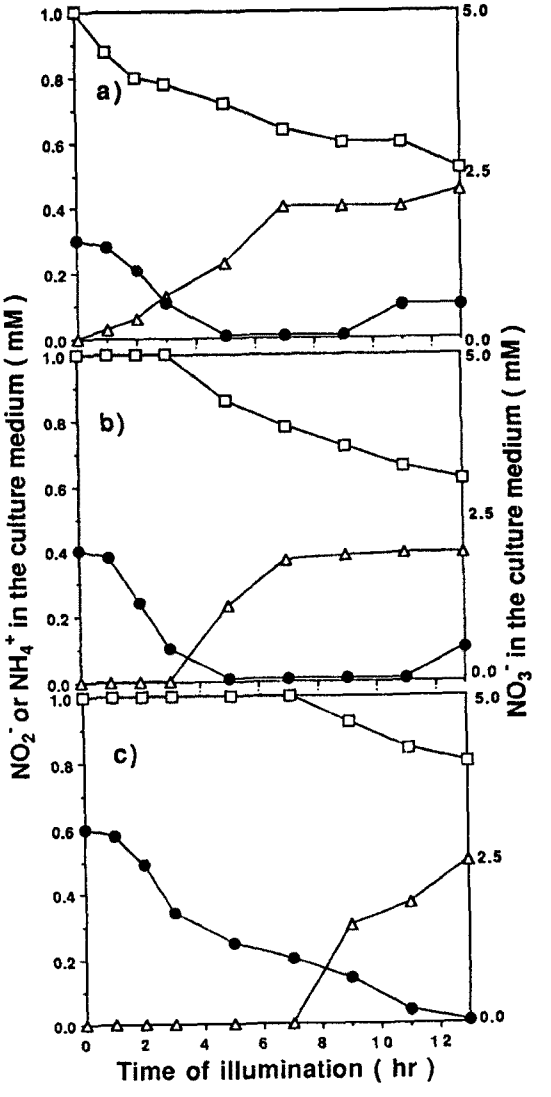

Fig. 3. Effect of both NO_2^- and NH_4^+ on NO_3^- utilization NO_3^- (□), NO_2^- (△) and NH_4^+ (●).
Cells were not dark-treated.

ing the progress of nitrate uptake (10). Ammonium may act as an allosteric effector, sharply diminishing the carrier affinity for nitrate on the outer side the membrane. This hypothesis explains the fact that nitrate uptake is not dependent on the concentraion of ammonium ion (1). However, results shown in Fig. 2 indicate that nitrate reductase activity in *B. braunii* depends on the ammonium ion concentration. That is nitrate reductase activity repressed at higher ammonium ion concentration than 0.2 mM.

Nitrate Uptake without Dark Treatment

Nitrate assimilation without dark treatment was also depend on ammonium concentration as shown in Fig. 3. Fig. 3(a) indicates nitrate reductase activity was not repressed by amount of 0.3 mM at initial concentration of ammonium, while it was initially repressed at higher than 0.3 mM of ammonium ion. But we failed to determine the accurate ammonium concentration which repressed nitrate use with the crude enzyme.

This may explain the fact that ammonium is not a direct allosteric inhibitor of nitrate reductase in this algal system.

Cell Death by Treatment of Ammonium Ion

When *B. braunii* exposed to 5 mM of ammonium for one day, *B. braunii* hardly assimilated any nitrate (Fig. 4). Unless treatment was done, nitrate was used

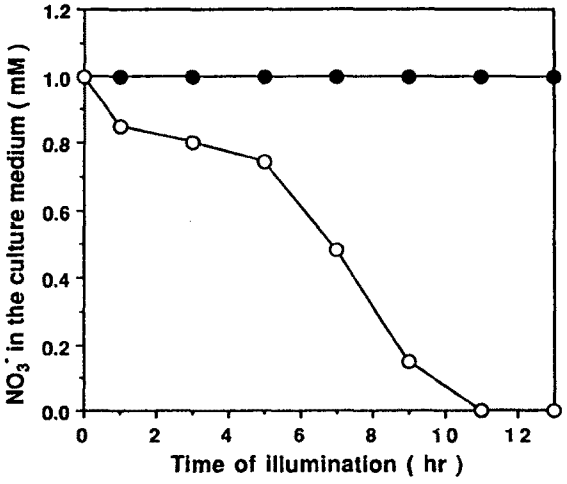

Fig. 4. Effect of ammonia treatment on nitrate utilization cells were exposed to 5 mM ammonia medium for 24 hours (●). No treated (○).
Cells were dark-treated before the illumination (see materials and methods).

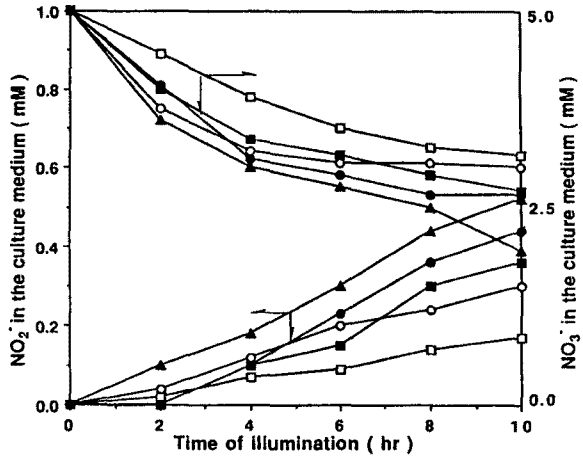

Fig. 5. Effect of pH on NO_3^- utilization and NO_2^- release pH 6 (□), pH 7 (○), pH 8 (▲), pH 9 (●) and pH 10 (■).
Cell were treated nitrogen starvation with nitrogen free medium for 6 hours.

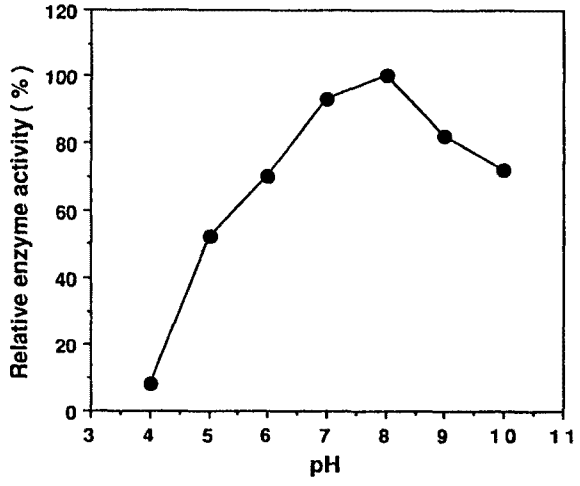

Fig. 6. Effect of pH on nitrate reductase activity.

over in 12 hrs. When *Chlorella* cells are exposed to ammonium for a long period (longer than 24 hours), the total activity of nitrate reductase, including the associated cytochrome *c* falls to the below 5% of the original level, which results a lowering of the rate of CO_2 fixation. Nitrate reductase, when stimulated by light, is assoiated with microbody-like organelles which attach themselves to chloroplasts during illumination (8). This is similiar to the activities of enzymes of CO_2 fixation (RuDP carboxylase) and photorespiration (glycolate oxidase). Moreover, since CO_2 fixation by the Calvin cycle is the ultimate source of the carbon skeleton for fixation of ammonium produced by assimilaory nitrate reduction, little of no fixation occur in the case of deactivation of nitrate reductase of cytochrome c reductase, as well as in the absence of CO_2. When the rate of CO_2 fixation is low the accumulation of potentially toxic intermediate products of nitrate assimilation, such as nitrite, hydroxamine and ammonium, would be prevented (11). In order to achieve a high CO_2 fixation rate a high activity of nitrate reductase, as well as a proper CO_2 concentration, is needed.

Effect of pH on Nitrate Use and Nitrase Reductase Activity

The pH of the culture medium also proved to be an important factor in the use of nitrate. Fig. 5 show that the pH optimum for nitrate consumption was 8 *in vivo*.

This result is supported by the optimum pH for nitrate reductase activity *in vitro* (Fig. 6). We have not yet proved that nitrate reductase activity is correlated with cell growth and hydrocarbon productivity. However, we have shown that optimum nitrate reductase activity is needed to maintain CO_2 fixation rate, and to avoid ammonium toxicity. Further studies on the effect of phosphate concentration, temperature and CO_2 concentration on the growth rate and hydrocarbon productivity of *Botryococcus braunii* are in progress.

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