ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Rhythm of Carbon and Nitrogen Fixation in Unicellular Cyanobacteria Under Turbulent and Highly Aerobic Conditions

S. Krishnakumar,[1] Sandeep B. Gaudana,[1] Ganesh A. Viswanathan,[1] Himadri B. Pakrasi,[2,3] Pramod P. Wangikar[1]

[1]Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India; telephone: 91-22-2576-7232; fax: 91-22-2572-6895; e-mail: wangikar@iitb.ac.in

[2]Department of Energy, Environmental, and Chemical Engineering, Washington University, St. Louis, MO

[3]Department of Biology, Washington University, St. Louis, MO

ABSTRACT: Nitrogen fixing cyanobacteria are being increasingly explored for nitrogenase-dependent hydrogen production. Commercial success however will depend on the ability to grow these cultures at high cell densities. Photo-limitation at high cell densities leads to hindered photoautotrophic growth while turbulent conditions, which simulate flashing light effect, can lead to oxygen toxicity to the nitrogenase enzyme. *Cyanothece* sp. strain ATCC 51142, a known hydrogen producer, is reported to grow and fix nitrogen under moderately oxic conditions in shake flasks. In this study, we explore the growth and nitrogen fixing potential of this organism under turbulent conditions with volumetric oxygen mass transfer coefficient (K_La) values that are up to 20-times greater than in shake flasks. In a stirred vessel, the organism grows well in turbulent regime possibly due to a simulated flashing light effect with optimal growth at Reynolds number of approximately 35,000. A respiratory burst lasting for about 4 h creates anoxic conditions intracellularly with near saturating levels of dissolved oxygen in the extracellular medium. This is concomitant with complete exhaustion of intracellular glycogen storage and upregulation of *nifH* and *nifX*, the genes encoding proteins of the nitrogenase complex. Further, the rhythmic oscillations in exhaust gas CO_2 and O_2 profiles synchronize faithfully with those in biochemical parameters and gene expression thereby serving as an effective online monitoring tool. These results will have important implications in potential commercial success of nitrogenase-dependent hydrogen production by cyanobacteria.

Biotechnol. Bioeng. 2013;110: 2371–2379.

KEYWORDS: cyanobacteria; nitrogen fixation; *Cyanothece*; flashing light; K_La; Reynolds number

The authors have no conflict of interest to declare.
Correspondence to: P. P. Wangikar
Contract grant sponsor: Department of Biotechnology, Ministry of Science and Technology, Government of India
Contract grant number: BT/Indo-Aus/04/04/2009
Contract grant sponsor: Consortium for Clean Coal Utilization at Washington University in St. Louis
Received 29 November 2012; Revision received 24 January 2013;
Accepted 20 February 2013
Accepted manuscript online 1 March 2013;
Article first published online 7 April 2013 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.24882/abstract)
DOI 10.1002/bit.24882

Introduction

Cyanobacteria, a group of photosynthetic prokaryotes, are believed to be responsible for the creation of oxic atmosphere on earth and are credited with 20–30% of the current global photosynthesis (Pisciotta et al., 2010; Waterbury et al., 1979). A number of cyanobacteria fix nitrogen in addition to carrying out oxygenic photosynthesis. Interestingly, nitrogenase, a key enzyme involved in nitrogen fixation, is rapidly and irreversibly inactivated on being exposed to molecular oxygen (Fay, 1992). As a result, nitrogen fixing cyanobacteria have evolved mechanisms to separate oxygen sensitive nitrogen fixation and oxygenic photosynthesis. These two processes are separated spatially in multi-cellular heterocystous cyanobacteria such as *Anabaena* sp. (Gallon, 1992) and temporally in unicellular diazotrophic cyanobacteria such as *Cyanothece* sp. (Schneegurt et al., 1994). Furthermore, nitrogen fixation is an energy intensive process which requires 16 molecules of ATP to fix one molecule of nitrogen into ammonia (Dean et al., 1993). In unicellular cyanobacteria, the energy generated by photosynthesis is stored as intracellular glycogen granules during the light phase, which is then released by oxidative breakdown of this storage molecule during the dark phase thereby generating the required energy for nitrogen fixation (Schneegurt et al., 1994). This respiratory phase is also believed to be responsible for generating the required oxygen deficient condition necessary for the activity of the nitrogenase enzyme.

The genus *Cyanothece* is comprised of unicellular, aerobic, and diazotrophic cyanobacteria. Strains of this genus have been isolated from diverse habitats ranging from intertidal areas to rice fields (Bandyopadhyay et al., 2011; Reddy et al., 1993). These organisms play a key role in nature's carbon and nitrogen cycles. *Cyanothece* sp. strain ATCC 51142 (henceforth *Cyanothece* 51142), a unicellular diazotroph, was isolated from the Texas gulf coast (Reddy et al., 1993). *Cyanothece* 51142 is known to produce hydrogen at levels that are highest amongst the known wild type photosynthetic microbes (Bandyopadhyay et al., 2010). This hydrogen production is believed to be associated with the nitrogenase enzyme activity. The genome of this organism has been sequenced (Welsh et al., 2008) and a large number of genes have been shown to oscillate under the control of a circadian clock (Stöckel et al., 2008; Toepel et al., 2008). Owing to these traits, *Cyanothece* 51142 has emerged as a model organism for studying photosynthetic biohydrogen production as well as studying circadian rhythm under nitrogen fixing condition.

To fully exploit the commercial potential of cyanobacteria such as *Cyanothece* sp., it would be imperative to be able to grow cultures at high cell densities. However, photoautotrophic cultures experience a self-shading effect resulting in uneven light distribution at high cell densities when grown with surface illumination (Carvalho et al., 2011; Han et al., 2000). Some cells remain exposed to light for long durations thereby experiencing photo-inhibition, while other cells remain in dark zones and experience photo-limitation (Han et al., 2000). These effects can be alleviated by introducing turbulence in the system which would simulate flashing light effect with rapid movement of cells between light and dark zones (Kliphuis et al., 2010; Sato et al., 2010). Flashing light has been reported as favorable mode of illumination for efficient photosynthesis (Dickson and Chua, 1963; Laws et al., 1983; Park and Lee, 2001). However, turbulence in vessel, which is necessary to create a flashing light effect, along with aeration can increase the oxygen tension in solution and this in turn may affect nitrogen fixation and growth under nitrogen fixing conditions. While *Cyanothece* 51142 has been grown under moderately oxic conditions in flasks and flat plate photobioreactors (Červený and Nedbal, 2009), growth has not been characterized at higher agitation and oxygen mass transfer rates. Under the present study we analyzed the influence of varying agitation rates on physiology and metabolism of *Cyanothece* 51142 grown under nitrogen fixing condition. Further, one of the aims of the study was to optimize the rate of agitation so as to facilitate optimal photosynthesis and nitrogen fixation.

Materials and Methods

Seed Culture

Cyanothece 51142 culture was grown in Erlenmeyer flasks in ASP2 medium with nitrate as reported earlier (Reddy et al., 1993). Briefly, the culture was grown under continuous light at an intensity of 50 μEinstein $m^{-2} s^{-1}$ in an orbital shaker-incubator at 120 revolutions per minute (rpm) and 30°C. The seed culture for inoculation in the bioreactor was grown in a 500 mL Erlenmeyer flask with nitrate containing ASP2 medium by using a 10% (v/v) inoculation of mid-exponential phase culture grown under continuous light. The seed culture was entrained in alternating light/dark phases of 12 h each for 3 days prior to inoculating the bioreactor. Flask to medium volume ratio was 2.5:1. Cell growth was monitored by measuring the optical density (OD) at 730 nm measured with Nanophotometer (Implen, München, Germany). The seed culture was then washed with nitrate-free ASP2 medium before inoculating the photo-bioreactor so as to obtain initial culture OD of approximately 0.3.

Photo-Bioreactor Setup

Axenic culture of *Cyanothece* 51142 was cultivated under nitrogen fixing conditions with a medium volume of 1.7 L in a Biostat B plus bioreactor fitted with 6-bladed disc impellers (Sartorius, Goettingen, Germany). Batch cultivations were carried out in nitrate deficient ASP2 medium as described earlier (Reddy et al., 1993). Four 40 W cool white fluorescent lamps (Oreva, Ahmedabad, India) were used to provide a surface light intensity of 90 μEinstein $m^{-2} s^{-1}$. The temperature of the reactor was maintained at 30°C. For aeration, 0.2 micron filtered atmospheric air was sparged from the bottom of the reactor at a flow rate of 0.1 volume/volume/min (vvm), maintained using a Rotameter. The exit gas from the reactor was analyzed with BlueInOne Cell exit gas analyzer (BlueSens, Herten, Germany), which recorded the volume percentages of CO_2 and O_2 at every 1 min interval. Likewise, dissolved oxygen and pH probes (Hamilton, Bonaduz, Switzerland) were used to measure the dissolved oxygen (DO) and pH values inside the bioreactor. Twenty-five milliliters of samples were drawn at pre-determined intervals to monitor growth, intracellular glycogen and chlorophyll contents and gene expression on the 5th day for 200 rpm and 600 rpm. Eight samples were drawn from a single diurnal cycle and are designated as L_1, $L_{4.33}$, and so on for the samples drawn 1 h in to the light cycle and 4.33 h in to the light cycle, respectively. Similarly D_1, $D_{4.33}$, and so on are designated for the samples from the dark cycle.

K_La Determination

The volumetric mass transfer coefficient for oxygen (K_La, h^{-1}) was determined by non-fermentative dynamic gassing out method (Nigam et al., 2012; Waites et al., 2001). Briefly, the medium was first sparged with nitrogen gas to remove the dissolved oxygen (DO). Air was then sparged at a constant flow rate of 0.1 vvm and the increase in the DO level was monitored using a DO probe (Hamilton, Bonaduz, Switzerland). The experiment was done in triplicates at

agitation speeds of 200, 400, 600, and 800 rpm. K_La values were calculated for all the individual agitation speeds as described by Equation (1)

$$\frac{dC_L}{dt} = K_La(C^* - C_L) \quad (1)$$

where C^* is the saturation concentration of DO while C_L is the concentration of DO in the medium.

Reynolds Number Calculation

Reynolds number was calculated to indicate the level of turbulence at the agitation speeds of 200, 400, 600, and 800 rpm as described by Equation (2) (Sinnott, 2005)

$$N_{Re} = \frac{\rho N D^2}{\mu} \quad (2)$$

where N_{Re} is the Reynolds number; ρ is the fluid density in $kg\,m^{-3}$; N is the impeller speed in s^{-1}; D is the impeller diameter in m, and μ is the fluid viscosity in $kg\,m^{-1}\,s^{-1}$.

Chlorophyll and Glycogen Estimation

Total chlorophyll 'a' was extracted in methanol and quantified spectrophotometrically as described earlier (Arnon et al., 1974; Colón-López et al., 1997). Briefly, 1 mL of methanol was added to the biomass pellet, incubated at 60°C in a water bath for 15 min, cooled to room temperature and centrifuged at 12,000*g*. Optical density of the supernatant was noted at 678, 750, and 620 nm and the amount of chlorophyll calculated. Intracellular glycogen content was estimated as previously reported with minor modifications (Bandyopadhyay et al., 2010). After methanol extraction of chlorophyll, 100 μl of 40% KOH was added to the resultant pellet and incubated at 95°C for 1 h to remove free glucose. Two hundred microliters of 100% ethanol was then added to this solution after cooling to room temperature and kept at −20°C overnight to precipitate glycogen. The samples were centrifuged for 1 h at 4°C at 12,000*g*. Forty microliters of 2 N HCl was added to the pellet and incubated at 95°C for 30 min. After cooling to room temperature, 40 μl of 2 N NaOH, 20 μl of 1.0 M phosphate buffer (pH 7.0), and 40 μl of distilled water were added and vortexed thoroughly. The modification involved estimation of the released glucose using a Glucose GOD PAP kit, a Glucose oxidase based enzyme assay kit (Biolab Diagnostics (I) Pvt. Ltd., Boisar, India) as per the manufacturer's instructions. Twenty microliters of this sample was mixed with 1 mL of glucose oxidase enzyme solution and incubated at 37°C for 15 min and the optical density was measured at 500 nm. The amount of glycogen in the sample was calculated from a standard plot.

Gene Expression Analysis

Total RNA was extracted using TRI reagent (Sigma–Aldrich, St. Louis, MO) as per the manufacturer's instructions. Briefly, cells equivalent to OD of 10.0 were harvested by centrifugation and washed with normal saline. One microgram of DNase treated total RNA was used for reverse transcription using Enhanced Avian RT First strand synthesis kit and random primers (Sigma–Aldrich). Gene specific primers (Table I) were designed to obtain an amplified product of 100–110 base pairs. Quantitative Real-Time PCR (qRT-PCR) was carried out using LuminoCt SYBR Green qPCR ReadyMix (Sigma–Aldrich) on LightCycler® 480 (Roche Diagnostics, Indianapolis, IN). Each reaction was carried out in four replicates, and the average C_T was used to calculate the ratios for quantifying the expression of a gene as compared with the control. The average expression of a gene across all time points was taken as control. The DNA contamination was ruled out for all samples by performing qRT-PCR on DNase treated but not reverse transcribed RNA samples.

Results and Discussion

Cyanothece 51142 has been reported to grow and fix N_2 under oxic conditions in shake flasks at agitation of 125 rpm. In 250–500 mL flasks, the light path length is of the order of

Table I. Description of genes and gene specific primer sequences used in the study.

Gene symbol	Gene id	Definition	Function of investigation	Primer sequences
nifH	cce_0559	Nitrogenase iron protein	N_2 fixation	F 5′-TACCATTGCTGCGTTAGCTG R 5′-TTAACCAAGGAGGCGGATTT
nifX	cce_0565	Nitrogen fixation protein	N_2 fixation	F 5′-GACCCCCATTAAAGCGAGAA R 5′-GTGCAGAATGGTGGTTTGTG
psaA	cce_0989	PS I P700 chlorophyll a apoprotein subunit Ia	Photosynthesis	F 5′-TGCCACCCTATCCCTACCAG R 5′-GGGCTCCAGCACCAACTATT
psbA1	cce_3501	PS II D1 protein	Photosynthesis	F 5′-ATCTTTATTCTCCGCTATGC R 5′-TCTTGTCCGAACTTGTAACCG
rrn16Sa	cce_RNA045	16S ribosomal RNA	Constitutive expression	F 5′-CCCTGGGCTACACACGTACT R 5′-TCTCGAGTTGCAGAGAGCAA

PS, photosystem.

1–2 cm with 1:5 medium to flask volume ratio. However, the light path length is much longer and of the order of several cm in stirred vessels and bubble columns with surface illumination. With an increase in the cell density, light penetration into interiors of vessels and columns gets compromised. This leads to dark zones and in turn photo-limitation for cells in such zones. Surface illumination combined with high agitation can help simulate flashing light effect, which has been deemed highly effective for efficient growth of photosynthetic organisms (Park and Lee, 2001). However, increased agitation would also lead to an increase in the oxygen tension in the solution. Therefore, our first objective was to study growth of *Cyanothece* under N_2 fixing conditions and under highly turbulent regimes in a stirred vessel. Shake flasks at agitation of 125 rpm provide volumetric oxygen mass transfer coefficient (K_La) of 7.2 h^{-1} (Zheng et al., 1998) (Table II). To simulate flashing light effect, we chose a stirred vessel with surface illumination of 90 μEinstein m^{-2} s^{-1} and at agitation speeds ranging from 200 to 800 rpm. These agitation speeds correspond to Reynolds number (N_{Re}) in the range of 11,700–46,800, respectively. Stirred vessels are considered to be operating under turbulent regime for N_{Re} values of >10,000 (Sinnott, 2005). Turbulent regime ensures rapid cyclic movement of the cells between the dark and light zones. Air was sparged continuously to supply CO_2 and remove O_2 from the medium during photosynthetic phase and to supply O_2 during the respiratory phase. Under the operating conditions, K_La for oxygen was in the range of 15–123 h^{-1} (Table II). It should be noted that the mass transfer rates corresponding to 600 and 800 rpm are substantially higher compared to the reported conditions used for this organism under nitrogen fixing condition (Bandyopadhyay et al., 2010; Červený and Nedbal, 2009; Reddy et al., 1993). We monitored growth of *Cyanothece* and online profiles of DO, pH, and exhaust gas CO_2 and O_2 concentrations for five diurnal periods when the culture was subjected to alternating light/dark regimes. The online measurements are known to indicate the physiological state of the culture and are useful in identifying shifts in the metabolic status (Bapat et al., 2006; Doan et al., 2007; Maiti et al., 2009).

Effect of Agitation on O_2 and CO_2 Evolution Rates

The oscillations in the O_2 and CO_2 evolution rates obtained in this study (Fig. 1) were similar to those reported for the dissolved O_2 and CO_2 profiles for this organism under nitrogen fixing condition in a flat cuvette photobioreactor (Červený and Nedbal, 2009). For instance, the CO_2 evolution rate decreased while the O_2 evolution rate increased at the onset of the light phase due to CO_2 fixation via oxygenic photosynthesis. Further, the gas profiles showed a characteristic respiratory burst with sharp rise and fall in the evolution rates of CO_2 and O_2, respectively, before the onset of dark phase (Fig. 1). This respiratory burst lasted for 3–4 h and may be a result of the requirements for energy and intracellular anoxic conditions during the night time nitrogen fixation. Notably, the dissolved oxygen concentration remained close to 100% of saturation even through the respiratory burst (data not shown) in contrast to a 30% reduction reported for flat plate photobioreactor (Červený and Nedbal, 2009). This suggests a significantly lower K_La for the flat plate PBR compared to that for a vessel at 200 rpm.

At an agitation speed of 200 rpm, the amplitudes of peaks for O_2 and CO_2 evolution rates (OER and CER, respectively) are the smallest amongst the agitation rates used in this study (Fig. 1). Further, the amplitudes dampen starting from the 3rd day indicating a photo-limitation imposed due to self shading. This limitation appears to be partially overcome with an increase in the speed of agitation to 400 rpm. However, the peaks for both OER and CER are optimal at 600 rpm, suggesting an optimal photosynthesis rate during the light phase and respiration rate during the dark phase. Further, the amplitudes of the respiratory burst progressively increased from Day 1 to Day 5 only at 600 rpm.

Table II. Volumetric mass transfer coefficient for O_2, K_La, and Reynolds number, N_{Re} for the air sparged and stirred vessel used in this study compared with that for shake flasks.

Growth geometry	Volumetric mass transfer coefficient for O_2, K_La (h^{-1})	Reynolds number[a] (N_{Re})	Reference
Shake flask (with cotton plug)			
1:5 volume ratio, 120 rpm	7.2	ND[b]	Zheng et al. (1998)
Air sparged and stirred vessel at 0.1 vvm aeration			
200 rpm	15.4 ± 0.1	11,600	Present study[c]
400 rpm	38.9 ± 0.6	23,300	Present study[c]
600 rpm	87.2 ± 0.4	35,000	Present study[c]
800 rpm	123 ± 0.1	46,000	Present study[c]

[a]Reynolds number was calculated for individual agitation speed as described in Materials and Methods Section.
[b]ND, not determined.
[c]Volumetric mass transfer coefficient of O_2 (K_La, h^{-1}) determined by dynamic gassing-out method for the stirred vessel at different agitation speeds at 0.1 vvm aeration. The K_La values are calculated from the slope by plotting $\ln(C^* - C_L)$ versus time (t), where C^* denotes the saturated dissolved oxygen concentration, in mmol dm^{-3} and C_L represents the concentration of dissolved oxygen in the medium, in mmol dm^{-3}.

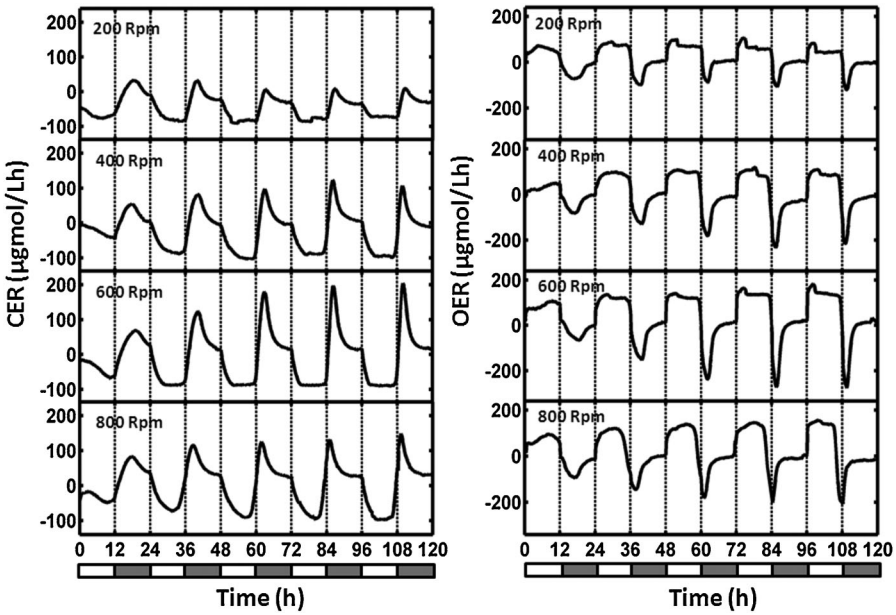

Figure 1. Carbon dioxide evolution rate (CER) and oxygen evolution rate (OER) observed during growth of *Cyanothece* sp. ATCC 51142 under nitrogen fixing conditions when subjected to alternating light and dark phases of 12 h each. The culture was grown in an externally illuminated, stirred-vessel bioreactor sparged with air at 0.1 volume/medium volume/min (vvm) and agitated at different speeds. Surface illumination of 90 µEinstein m^{-2} s^{-1} was used. The gas evolution rates are calculated based on the difference in feed and exhaust concentrations of CO_2 and O_2 in the gas streams. The light and dark phases are depicted as empty and gray shaded regions, respectively, on the bar below the time axis. The vertical grids correspond to the light switching times. Representative profiles from two biological replicates per condition are shown.

The sub-optimal growth and OER and CER rates at 800 rpm may result from too quick a movement from the surface, high oxygen mass transfer rates or shear rates or a combination thereof.

Effect of Agitation on Growth

On comparison of the growth profiles for all the agitations speeds, agitation at 400 and 600 rpm showed better growth than that at 200 and 800 rpm (Fig. 2). In case of 200 rpm, the culture could not sustain the rate of growth after the second day possibly due to photo-limitation caused by self-shading. At 800 rpm, there appeared to be an initial lag in growth, followed by which the growth was comparable to that at 200 rpm. In all cases, the growth was noticeably higher than in a shake flask at 125 rpm under otherwise similar conditions (data not shown). Further, the cells remained in suspension with no signs of clumping and with normal morphology at all the agitation speeds (data not shown). This result shows that *Cyanothece* cells grow and fix nitrogen in highly oxic environment at high oxygen tensions in the medium.

Effect of Agitation on Photosynthesis and Respiration Activity

The plots of d(CER)/d*t* and d(OER)/d*t*, the first derivatives of the gas evolution rates, provide clues regarding shifts from photosynthetic to respiratory metabolic phases and vice versa (Fig. 3). To exemplify, a sharp positive peak in d(OER)/d*t* at the onset of light is indicative of the onset of photosynthetic activity while the negative peak just before the dark indicates onset of respiration. The shift from respiration to photosynthesis occurs over a period of few minutes as compared to 2–3 h for the reverse shift as

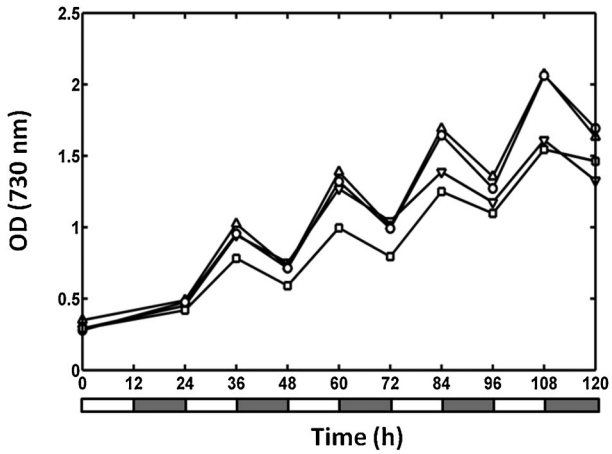

Figure 2. Growth profile of *Cyanothece* sp. ATCC 51142 at agitation speeds of 200 rpm (▽), 400 rpm (Δ), 600 rpm (○), and 800 rpm (□) when grown under nitrogen fixing conditions. Other growth conditions as described in legend to Figure 1.

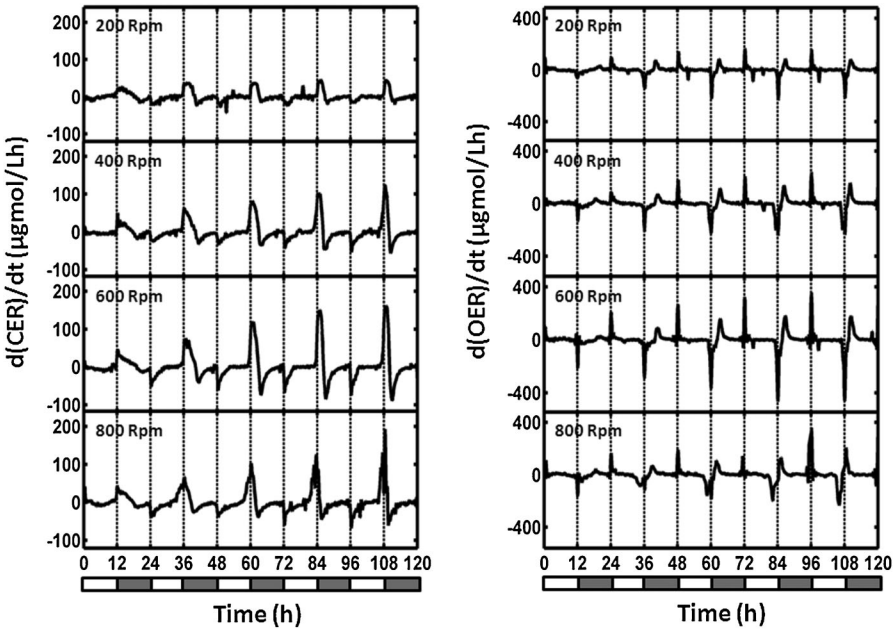

Figure 3. Rate of change of evolution rates of CO_2 (d(CER)/d*t*) and O_2 (d(OER)/d*t*) during the growth of *Cyanothece* sp. ATCC 51142 under nitrogen fixing conditions for different agitation speeds. The rates are obtained as first derivative of the results shown in Figure 1. Experimental details as described in legend to Figure 1.

indicated by the broad peaks around the light switch-off time. Thus, it was of interest to further analyze the shift in metabolism around the light switch-off time. We therefore compare the O_2 profiles 6 h before and after the light switch-off time for the different agitation speeds. We observe that the response in CER lags behind that in OER possibly due to greater solubility of CO_2 compared to O_2 and hence only O_2 profiles were considered for this analysis (Fig. 4). The profile for 400 rpm from the figure was excluded for brevity.

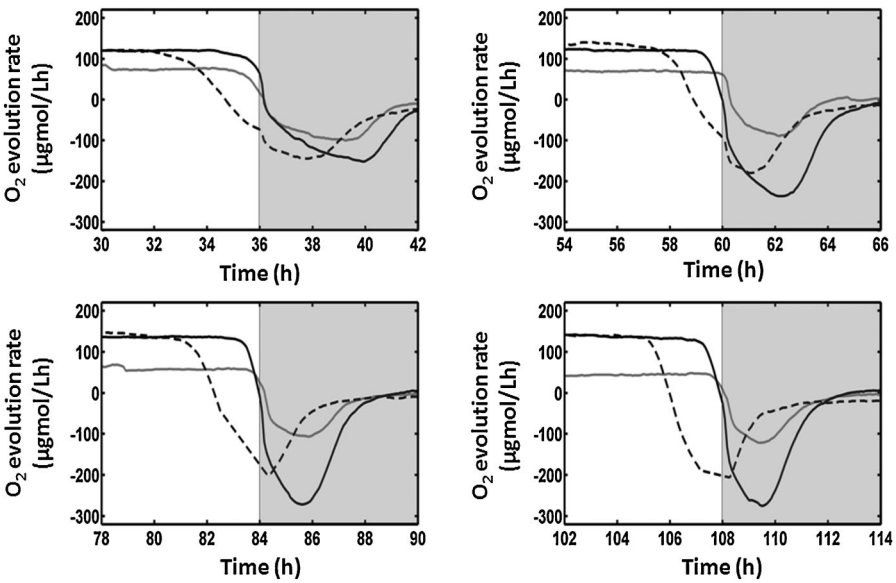

Figure 4. A view of the profiles of oxygen evolution rates shown in an enlarged manner around the light switch-off times on days 2–5. Solid lines in gray and black correspond to agitation speeds of 200 and 600 rpm, respectively, while the dashed line corresponds to 800 rpm. Data are shown for 6 h before and 6 h after the lights were switched off. Experimental details as described in legend to Figure 1.

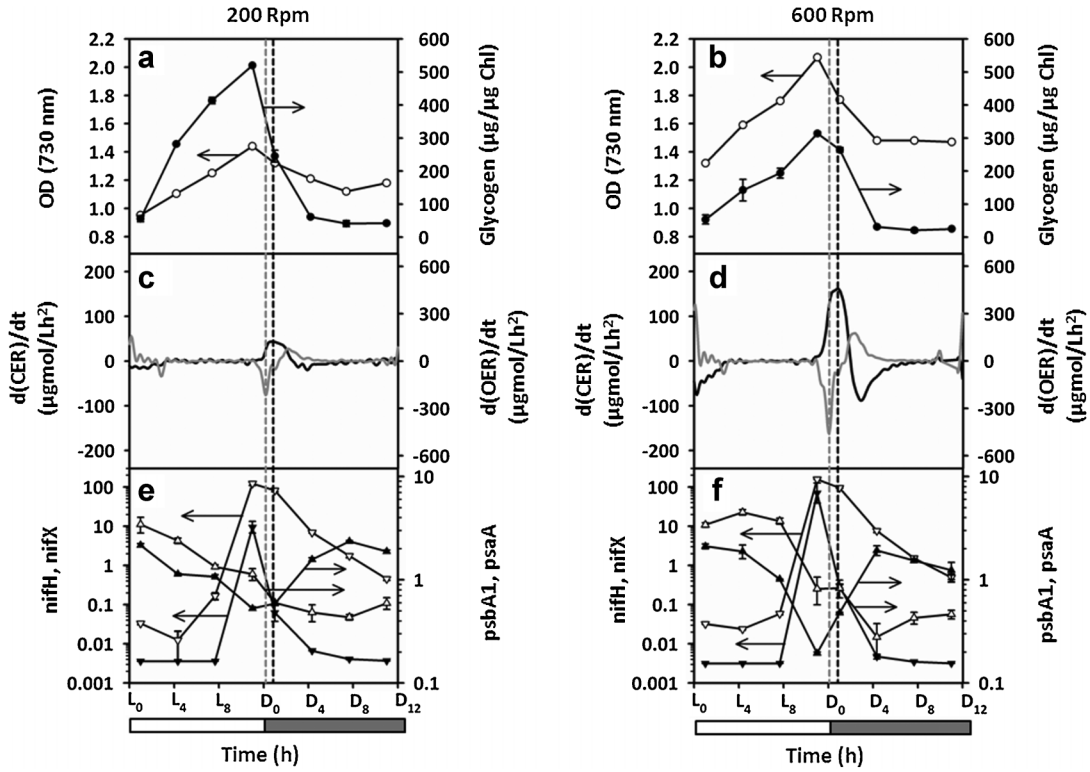

Figure 5. Profiles of glycogen content and gene expression in the 5th diurnal period for the growth of *Cyanothece* sp. ATCC 51142 at agitation speeds of 200 rpm (panels a, c, and e) and 600 rpm (panels b, d, and f). Symbols (●) and (○) in the panels a and b indicate, respectively, the glycogen concentration and optical density of the organism. Subsequent panels c and d show the rate of change of CO_2 (black) and O_2 (gray) evolution rates of 200 rpm and 600 rpm, respectively. In this, the dashed vertical line from the peak rise (d(CER)/d*t*) in black color and from peak fall (d(OER)/d*t*) in gray color are projected to all the panels to serve as a reference line. The last panels e and f show the fold change gene expression values of nitrogen fixing and photosynthetic genes. Symbols (▽), (▼), (Δ), and (▲) indicate the fold change in expression of *nifH, nifX, psbA1*, and *psaA*, respectively. The values in panels a, b, e, and f are mean from four replicates, error bars indicate the standard deviation. Other experimental details as described in legend to Figure 1.

From the OER profile, we notice that 800 rpm exhibited an early respiratory burst for all the 5 days, possibly to manage the increased oxic condition under such high agitation speed (Fig. 4). Thus, the photosynthesis phase seems to get significantly shortened at 800 rpm and the culture does not seem to be able to make full use of the light phase for photosynthesis. The onset of respiratory burst occurred later at 600 rpm than at 800 rpm but before the onset of the dark phase. On the other hand, 200 rpm showed no signs of early respiratory burst and the shift to respiratory phase occurred very close to the onset of dark.

Oscillations in Glycogen Content

Cyanothece 51142 stores energy in the form of intracellular glycogen granules during the photosynthesis phase (Schneegurt et al., 1994). We observed that the shift from photosynthesis to respiration takes place approximately 1 h before the onset of dark phase. Further, the respiratory burst was observed to last for about 3–4 h. Thus, it was of interest to ascertain if the glycogen that builds up during the 11 h of photosynthesis would get consumed during the respiratory burst. In the light phase, from L_1 to L_{11}, the glycogen concentration gradually increased with the highest concentration attained at L_{11} (Fig. 5a and b). Thereafter, there was a sharp decline in the glycogen content in the next 5 h. This indicates that the synthesized glycogen granules were catabolized during the respiratory burst to yield the required energy for the N_2 fixation activity. These broadly agree with the trends in glycogen content as reported earlier (Schneegurt et al., 1994). Interestingly, the quantified glycogen and OD profiles for both 200 and 600 rpm followed a similar oscillation trend as the exit gas CO_2 and O_2 profiles.

Oscillations in Gene Expression Profile

To capture the impact of the timing of respiratory burst on the expression of genes of the nitrogenase complex, we monitored the expression of *nifH* and *nifX* genes. These genes are important representatives from *nif* operon (Dean et al., 1993; Memon et al., 2013) and the upregulation of

these genes is in turn indicative of the preparation of the cells for synthesis of Nitrogenase enzyme for the process of nitrogen fixation. It was hypothesized that we may observe a delay in the peaking of *nif* genes in case of 200 rpm than that in 600 rpm. However, with our frequency of sampling, we observed that peak in *nifH* and *nifX* expression occurs at L_{11}, that is 1 h prior to the onset of dark cycle. On the other hand, the amplitude for the genes' expressions was higher in case of 600 rpm. At 600 rpm agitation, *nifH* and *nifX* genes showed a highest fold change in expression of 157-fold for both the genes at time point L_{11} (Fig. 5f). In case of 200 rpm the highest fold change in expression of *nifH* and *nifX* genes was 123- and 111-fold, respectively, which was also at time point L_{11} (Fig. 5e). The samples from the 200 rpm and 600 rpm were also analyzed for the expression of *psaA* and *psbA1* genes to study the effect of agitation rates on photosynthesis genes. In general the pattern to expression of these genes was observed as reported earlier (Colón-López and Sherman, 1998), with *psaA* building up in dark and *psbA1* showing increasing expression in light cycle and declining in the dark. The expression levels of *psbA1* remain high in the light cycle with peak expression of 4.5-fold observed at $L_{4.33}$ for 600 rpm (Fig. 5f) and that of 3.4-fold at L_1 for 200 rpm (Fig. 5e). The expression levels for *psaA* on the other hand increase during dark and fall in the light cycle, with peak observed at L_1 with about twofold change for both 600 rpm and 200 rpm. The higher level of expression of *psbA1* in 600 rpm as compared to 200 rpm was consistent with *nif* gene expression and other analyzed parameters. More importantly, the higher level of gene expression for *psbA1* also sustained longer at 600 rpm, which show high fold expression till 4.33 h into the light cycle. In case of 200 rpm however, *psbA1* gene expression significantly falls by the time of 4.33 h in to light cycle. The expression patterns of *psaA* and *psbA1* genes jointly reemphasize what is also indicated from the online parameters and other analysis, that at 200 rpm the cells might be experiencing a limitation of light owing to the self-shading and hence there is an early onset of increase and decrease of the *psaA* and *psbA1* genes, respectively. This limitation seems to be overcome at 600 rpm.

Conclusions

The organism grows well in a stirred vessel under turbulent regime with optimal growth at Reynolds number of approximately 35,000. Turbulent regime ensures rapid circulation of cells between the dark and light zones thereby simulating flashing light effect. The growth under optimal conditions is more than twofold than that in a shake flask. Interestingly, the organism thrives under highly oxic conditions with K_La values that are approximately 12-fold compared to those in shake flask. This adds an exciting paradigm to the existing understanding of nitrogen fixation and more importantly indicates a highly competent oxygen scavenging and exclusion mechanism(s) for this organism. The study suggests a simple physical means of enhancing growth of *Cyanothece* 51142 under nitrogen fixing conditions. Furthermore, the synchronization between the exit gas and the biochemical and gene expression profiles suggest that the exit gas profile is a reliable indication of the metabolic state of the cells. Hence we propose that the analysis of the exit gas profiles can be employed as quick and convenient method for studying metabolic rhythms. The results have important implications as nitrogen fixing cyanobacteria are being considered for hydrogen production.

This work was partially supported by grants from Department of Biotechnology, Ministry of Science and Technology, Government of India (grant number BT/Indo-Aus/04/04/2009) awarded to P.P.W. and from the Consortium for Clean Coal Utilization at Washington University in St. Louis awarded to P.P.W. and H.B.P. We are thankful to the anonymous reviewers for their valuable suggestions.

References

Arnon DI, McSwain BD, Tsujimoto HY, Wada K. 1974. Photochemical activity and components of membrane preparations from blue green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. Biochim Biophys Acta 357(2):231–245.

Bandyopadhyay A, Elvitigala T, Welsh E, Stöckel J, Liberton M, Min H, Sherman LA, Pakrasi HB. 2011. Novel metabolic attributes of the genus *Cyanothece*, comprising a group of unicellular nitrogen-fixing cyanobacteria. mBio 2(5):e00214–11.

Bandyopadhyay A, Stöckel J, Min H, Sherman LA, Pakrasi HB. 2010. High rates of photobiological H 2 production by a cyanobacterium under aerobic conditions. Nat Commun 1:139.

Bapat PM, Das D, Dave NN, Wangikar PP. 2006. Phase shifts in the stoichiometry of rifamycin B fermentation and correlation with the trends in the parameters measured online. J Biotechnol 127(1):115–128.

Carvalho AP, Silva SO, Baptista JM, Malcata FX. 2011. Light requirements in microalgal photobioreactors: An overview of biophotonic aspects. Appl Microbiol Biotechnol 89(5):1275–1288.

Červený J, Nedbal L. 2009. Metabolic rhythms of the cyanobacterium *Cyanothece* sp. ATCC 51142 correlate with modeled dynamics of circadian clock. J Biol Rhythms 24(4):295–303.

Colón-López MS, Sherman DM, Sherman LA. 1997. Transcriptional and translational regulation of nitrogenase in light- dark- and continuous-light-grown cultures of the unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142. J Bacteriol 179(13):4319–4327.

Colón-López MS, Sherman LA. 1998. Transcriptional and translational regulation of photosystem I and II genes in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142. J Bacteriol 180(3):519–526.

Dean DR, Bolin JT, Zheng L. 1993. Nitrogenase metalloclusters: Structures, organization, and synthesis. J Bacteriol 175(21):6737–6744.

Dickson MH, Chua SE. 1963. Effect of flashing light on plant growth rate. Nature 198(4877):305.

Doan XT, Srinivasan R, Bapat PM, Wangikar PP. 2007. Detection of phase shifts in batch fermentation via statistical analysis of the online measurements: A case study with rifamycin B fermentation. J Biotechnol 132(2):156–166.

Fay P. 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56(2):340–373.

Gallon JR. 1992. Reconciling the incompatible: N_2 fixation and O_2. New Phytol 122(4):571–609.

Han BP, Virtanen M, Koponen J, Straškraba M. 2000. Effect of photoinhibition on algal photosynthesis: A dynamic model. J Plankton Res 22(5):865–885.

Kliphuis AMJ, de Winter L, Vejrazka C, Martens DE, Janssen M, Wijffels RH. 2010. Photosynthetic efficiency of *Chlorella sorokiniana* in a turbulently mixed short light-path photobioreactor. Biotechnol Prog 26(3):687–696.

Laws EA, Terry KL, Wickman J, Chalup MS. 1983. Simple algal production system designed to utilize the flashing light effect. Biotechnol Bioeng 25(10):2319–2335.

Maiti SK, Srivastava RK, Bhushan M, Wangikar PP. 2009. Real time phase detection based online monitoring of batch fermentation processes. Process Biochem 44(8):799–811.

Memon D, Singh AK, Pakrasi HB, Wangikar PP. 2013. A global analysis of adaptive evolution of operons in cyanobacteria. Antonie Van Leeuwenhoek 103(2):331–346.

Nigam A, Phale PS, Wangikar PP. 2012. Assessment of the metabolic capacity and adaptability of aromatic hydrocarbon degrading strain *Pseudomonas putida* CSV86 in aerobic chemostat culture. Bioresour Technol 114:484–491.

Park KH, Lee CG. 2001. Effectiveness of flashing light for increasing photosynthetic efficiency of microalgal cultures over a critical cell density. Biotechnol Bioprocess Eng 6(3):189–193.

Pisciotta JM, Zou Y, Baskakov IV. 2010. Light-dependent electrogenic activity of cyanobacteria. PLoS ONE 5(5):e10821.

Reddy KJ, Haskell JB, Sherman DM, Sherman LA. 1993. Unicellular, aerobic nitrogen-fixing cyanobacteria of the genus *Cyanothece*. J Bacteriol 175(5):1284–1292.

Sato T, Yamada D, Hirabayashi S. 2010. Development of virtual photobioreactor for microalgae culture considering turbulent flow and flashing light effect. Energy Convers Manage 51(6):1196–1201.

Schneegurt MA, Sherman DM, Nayar S, Sherman LA. 1994. Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. J Bacteriol 176(6):1586–1597.

Sinnott RK. 2005. Coulson and Richardson's chemical engineering, Volume 6—Chemical engineering design, 4th edn. Burlington, MA: Butterworth-Heinemann. 73 p.

Stöckel J, Welsh EA, Liberton M, Kunnvakkam R, Aurora R, Pakrasi HB. 2008. Global transcriptomic analysis of *Cyanothece* 51142 reveals robust diurnal oscillation of central metabolic processes. Proc Natl Acad Sci U S A 105(16):6156–6161.

Toepel J, Welsh E, Summerfield TC, Pakrasi HB, Sherman LA. 2008. Differential transcriptional analysis of the cyanobacterium *Cyanothece* sp. strain ATCC 51142 during light–dark and continuous-light growth. J Bacteriol 190(11):3904–3913.

Waites MJ, Morgan NL, Rockey JS, Higton G. 2001. Industrial microbiology: An introduction. Blackwell Science Ltd. p. 99–101.

Waterbury JB, Watson SW, Guillard RRL, Brand LE. 1979. Widespread occurrence of a unicellular, marine, planktonic, cyanobacterium. Nature 277(5694):293–294.

Welsh EA, Liberton M, Stöckel J, Loh T, Elvitigala T, Wang C, Wollam A, Fulton RS, Clifton SW, Jacobs JM, Aurora R, Ghosh BK, Sherman LA, Smith RD, Wilson RK, Pakrasi HB. 2008. The genome of *Cyanothece* 51142, a unicellular diazotrophic cyanobacterium important in the marine nitrogen cycle. Pro Natl Acad Sci U S A 105(39):15094–15099.

Zheng ZG, Liu D, Hu ZB. 1998. Comparison of cell growth and alkaloid production of Catharanthus roseus cells cultured in shake flask and in bioreactor. Acta Bot Sin 40(1):51–55.