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Demonstration of a two-stage aerobic/anaerobic chemostat for the enhanced production of hydrogen and biomass from unicellular nitrogen-fixing cyanobacterium

Pongsathorn Dechatiwongse, Geoffrey Maitland, Klaus Hellgardt *

Department of Chemical Engineering, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom

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ABSTRACT

Due to incompatible demands between the cyanobacterial growth and its sequential hydrogen (H_2) photoproduction mechanism, a two-stage chemostat photobioreactor (PBR) system was employed to cultivate a unicellular, nitrogen-fixing cyanobacterium *Cyanothece* sp. ATCC 51142. Our developed system, consisting of a series of two physically separated PBRs, has been operated non-stop for consecutive 750 h (~31 days), without any losses in its performance. Based on nutrient kinetics determined in batch PBR, a dilution rate of 0.015 h^{-1} was chosen to replenish the culture with all of essential substrates, while not interrupting the H_2 formation reaction. The physiological steady-state condition was achieved on day 12. The dry biomass concentrations, accumulated in the primary growth and the secondary H_2-production PBRs, reached the final values of 2 and 1.5 g L^{-1} respectively. By comparing to a single-stage batch system, the chemostat displayed more than 6.4 times higher H_2 productivity and 7.3 folds greater biomass yield. The steady-state conversion from glycerol into H_2 was ~28.3%. The stationary growth phase was also observed when the nutrient-replete culture eventually became under light-limited condition. Additionally, glycerol was reproducibly demonstrated as an effective anaerobic-inducer onto an air-incubated culture of this *Cyanothece* strain.

Nomenclature

Ar	Argon inert gas
ASP2	Artificial seawater medium
Cyanothece 51142	Unicellular cyanobacterium *Cyanothece* sp. ATCC 51142
$[C_i(t)]$	Concentration of nutrient "i" at any specific time "t"
$[C_{total,\ i}]$	Total consumption of nutrient "i"
$[C_{f,\ i}]$	Final concentration of nutrient "i"
Chl	Chlorophyll
CO_2	Molecular carbon dioxide
D	Dilution rate
D_c	Critical dilution rate
DD	Continuous dark condition
H_2	Molecular hydrogen
He	Helium inert gas
HVPs	High-value products
I	Light intensity
IC	Ion Chromatography
K_s	Half-velocity constant
LL	Continuous light condition
LD	Alternating light–dark condition
O_2	Molecular oxygen
OD(t)	Optical density at any specific time "t"
OD_{max}	Maximum optical density
PBR	Photobioreactor
PO_4^{-3}	Phosphate anion
pO_2	Dissolved oxygen concentration
MIMS	Membrane-inlet mass spectrometry
N_2	Molecular nitrogen
NO_3^{-1}	Nitrate anion
NH_3	Ammonia
NMR	Nuclear magnetic resonance
r	Nutrient uptake rate
S	Substrate concentration
SO_4^{-2}	Sulphate anion
T	Temperature
t_0	Inflexion point of the function
X	Dry biomass concentration
Y_{XS}	Yield coefficient
μ	Specific growth rate
μ_{max}	Maximum specific growth rate

* Corresponding author.
E-mail address: k.hellgardt@imperial.ac.uk (K. Hellgardt).

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1. Introduction

1.1. Cyanobacterial H_2 production

Hydrogen (H_2) has long been promoted as a potential energy carrier for future sustainable communities, due to its clean combustion and high energy density per unit mass [1]. The concept of solar-driven H_2 production using photobiological systems has been studied and reviewed [2–4] for some time as one of the most promising routes to sustainable generation of this gaseous product. Cyanobacteria, also known as blue-green algae, are a genetically diverse group of gram-negative photoautotrophic bacteria, whose habitats are widespread in fresh water, marine and terrestrial ecosystems. They are regarded as important contributors to the oxygenation of the global atmosphere, which in turn stimulated the evolution of plants and animals on the Earth [5]. In addition to O_2 evolution, cyanobacteria are also able to produce H_2 via two distinct pathways [6–8]. The first route, catalysed by hydrogenase enzymes, is a result of the recombination of protons and electrons obtained from the photosynthetic water-splitting activity of photosystem II (PSII). Another possible route, catalyse by nitrogenase enzymes, is the fixation of atmospheric nitrogen (N_2) to form ammonia (NH_3), during which H_2 is evolved as by-product. As both enzymes are severely inhibited in the presence of O_2 [9], the formation of H_2 is generally observed under microanaerobic conditions. In the natural environment, cyanobacteria are reported to employ two different approaches to protect their enzymes from being deactivated. One, demonstrated by a group of filamentous cyanobacteria e.g. *Anabaena* and *Nostoc*, is to differentiate 5–10% of their filamentous cells into specialised O_2-free compartments, called "heterocysts" under starvation of nitrogen [7,10,11]. Another approach, seen from a group of non-heterocystous cyanobacteria e.g. *Cyanothece*, *Gelothece* and *Oscillatoria*, is to temporally separate two incompatible metabolic processes – O_2-sensitive N_2-fixation and O_2-evolving photosynthesis – into different time periods [9,12,13].

1.2. Drawbacks of batch biological H_2 photoproduction systems

Despite the number of studies conducted over the past decades [2, 14], biophotolytic hydrogen production has failed to move to commercial scale up stages, due to i) the low productivity of microbial H_2-producers as well as ii) their incapability to generate the gas for a long period of operation.

It is possible to enhance the H_2 production rate by incubating cultures with inert gases (usually Ar), as this provides the (temporary) anaerobic environment, which is favourable for the activity of H_2-catalytic enzymes. One example (see Table 1) is the significant rate enhancements observed (almost 2-fold increase in rate), when cultures of two cyanobacteria *Cyanothece* sp. ATCC 51142 (referred as *Cyanothece* 51142) [15] and *Anabaena variabilis* PK84 [16] were incubated in Ar instead of air. However, when it comes to the scale up of biohydrogen processes, such external interference is likely not to be a feasible option, as it would lead to a more complex processing operation together with higher associated costs. It is thus more desirable to develop novel and robust but inexpensive techniques and/or process designs, which can internally induce an anaerobiosis upon air-incubated cultures. One commonly employed technique is nutrient starvation. For example, sulphur-deprivation of the green alga *Chlamydomonas reinhardtii* [17–19] is found to effectively prohibit light-induced photosynthetic O_2 evolution, but does not affect cellular respiration. Under these conditions dissolved O_2 eventually becomes depleted and H_2 starts to evolve, albeit for a short period of up to 120 h [20]. Despite its effectiveness of starting the gas-producing mechanism, this deprivation technique applies extreme biochemical stress to the cells and drives them towards the death phase. In response to experiencing anaerobic condition, microorganisms are also known to excrete a number of inhibitory by-products — organic acids as well as CO_2, which possesses an acidic character and subsequently causes undesirable physiological conditions in the culture [21,22]. As a result, it can be concluded from Table 1 that, regardless of species being cultivated in batch bioreactors, the gas

Table 1
H_2 productivity observed from some model green algae and cyanobacteria (wild-type as well as mutant) in batch and chemostat cultivation systems.

Microorganism	Microorganism description	H_2 productivity (μmol H_2 mg Chl^{-1} h^{-1})	Description of growth and H_2 production conditions	References
Chlamydomonas reinhardtii	Wild-type, fresh water, unicellular green alga	Batch cultivation 6.4 ± 0.36	Within 50 h after sulphur-deprivation, the production rate reached its maximum value and started to decline 10 h later.	[19]
		Chemostat cultivation 1.7[a]	Two-stage chemostat PBRs. Non-stop operation for more than 4,000 h (~5.5 months). LL growth and H_2. Sulphur-deprivation was employed to induce anaerobiosis.	[25]
Nostoc flagelliforme	Wild-type, N_2-fixing, heterocystous cyanobacterium	Batch cultivation ~12 .0 ± 1.5	Aerobic growth. 24 days of cultivation. 1 day after the inoculation, maximum H_2 production rate being observed and then suddenly declined.	[26]
		Chemostat cultivation 84.2 ± 2.3[a]	A cylindrical chemostat PBR. Non-stop operation for more than 72 days. LL growth and H_2.	[26]
Anabaena variabilis PK84	Mutant, N_2-fixing heterocystous cyanobacterium	Batch cultivation 106 ± 7.0	Automated helical PBR. Stimulated outdoor condition — LD and alternating temperature. Air incubation.	[16]
		191.0 ± 11	Automated helical PBR. Stimulated outdoor condition - LD and alternating temperature. Ar incubation.	
Cyanothece sp. ATCC 51142	Unicellular, marine, N2-fixing, non-heterocystous cyanobacterium	Batch cultivation 33 ± 5	LL growth and H_2. Air incubation. Glycerol supplement. Maximum gas production rate observed between 24 and 36 h after the incubation.	[27]
		225 ± 5	LD growth and LL H_2. Air incubation. Glycerol supplement.	[15]
		Up to 467	LD growth and LL H_2. Ar incubation. Glycerol supplement.	

Continuous light — LL. Alternating light dark — LD.
[a] Indicates that the system was perturbated with different conditions, but only the optimal value is presented here.