Advances in Biochemistry and Biotechnology

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Preface

The text *Advances in Biochemistry and Biotechnology* presents current trends in modern biotechnology. The aim is to cover all aspects of this interdisciplinary technology where knowledge, methods and expertise are required from chemistry, biochemistry, microbiology, genetics, chemical engineering and computer science. Evolutionary and biotechnological implications of robust hydrogenase activity in halophilic strains of tetraselmis have been focused in first chapter. Second chapter focuses on gene cloning, protein expression and biotechnological applications of venomous animals. In third chapter, we argue that the nucleic acid ligases from Archaea represent a largely untapped pool of enzymes with diverse and potentially favorable properties for new and emerging biotechnological applications. Biotechnological tools for garlic propagation and improvement have been described in fourth chapter. Fifth chapter considers a statistical model to describe the spatial dynamics of firm size across the biotechnology industry. Sixth chapter deals with biotechnology of agricultural wastes recycling through controlled cultivation of mushrooms. A brief review on lignocellulosic biomass and cellulases has been presented in seventh chapter. Application of green fluorescent protein in immunoassays has been introduced in eighth chapter. Last chapter proposes the use of the phage-display technique as a tool to identify new reagents that may be effectively used in immunological assays

Contents

Chapter 1

EVOLUTIONARY AND BIOTECHNOLOGICAL IMPLICATIONS OF ROBUST HYDROGENASE ACTIVITY IN HALOPHILIC STRAINS OF TETRASELMIS

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ABSTRACT

Although significant advances in H_2 photoproduction have recently been realized in fresh water algae (e.g. Chlamydomonas reinhardtii), relatively few studies have focused on H_2 production and hydrogenase adaptations in marine or halophilic algae. Salt water organisms likely offer several advantages for biotechnological H_2 production due to

the global abundance of salt water, decreased H_2 and O_2 solubility in saline and hypersaline systems, and the ability of extracellular NaCl levels to influence metabolism. We screened unialgal isolates obtained from hypersaline ecosystems in the southwest United States and identified two distinct halophilic strains of the genus Tetraselmis (GSL1 and QNM1) that exhibit both robust fermentative and photo H_2-production activities. The influence of salinity (3.5%, 5.5% and 7.0% w/v NaCl) on H_2production was examined during anoxic acclimation, with the greatest in vivo H_2-production rates observed at 7.0% NaCl. These Tetraselmis strains maintain robust hydrogenase activity even after 24 h of anoxic acclimation and show increased hydrogenase activity relative to C. reinhardtii after extended anoxia. Transcriptional analysis of Tetraselmis GSL1 enabled sequencing of the cDNA encoding the FeFe-hydrogenase structural enzyme (HYDA) and its maturation proteins (HYDE, HYDEF and HYDG). In contrast to freshwater Chlorophyceae, the halophilic Tetraselmis GSL1 strain likely encodes a single HYDA and two copies of HYDE, one of which is fused to HYDF. Phylogenetic analyses of HYDA and concatenated HYDA, HYDE, HYDF and HYDG in Tetraselmis GSL1 fill existing knowledge gaps in the evolution of algal hydrogenases and indicate that the algal hydrogenases sequenced to date are derived from a common ancestor. This is consistent with recent hypotheses that suggest fermentative metabolism in the majority of eukaryotes is derived from a common base set of enzymes that emerged early in eukaryotic evolution with subsequent losses in some organisms.

INTRODUCTION

The phylogenetically unrelated NiFe- and FeFe-hydrogenases have convergently evolved to catalyze the reversible reduction of protons to H_2 ($2H^+ + 2e^- <=> H_2$) [1]. Several recent studies have documented the diversity of hydrogenase-encoding genes in environments that span a broad range of geochemistry [2]–[8]. In some systems, e.g., terrestrial or marine hydrothermal communities, H_2 oxidation has been suggested to represent the primary mechanism of energy conservation [9], [10]. Yet, in other systems, e.g., terrestrial or intertidal phototrophic communities, H_2 evolution appears to be of critical importance to the functioning of the assemblage, in particular

at night when the systems become net sources of H_2 [11]–[13].

Biological H_2 production requires low-potential reducing equivalents derived from either fermentative pathways that oxidize fixed carbon (typically carbohydrates), or from photosynthetic pathways [14]–[19]. Several eukaryotic algae generate fermentative H_2 during dark, anoxic acclimation as part of a suite of fermentative pathways that catabolize carbohydrates to alcohols, organic acids and H_2, which are secreted. These metabolites likely provide a rich source of carbon building blocks and reducing equivalents to organisms inhabiting ecological niches adjacent to the algae, which are responsible for the majority of primary productivity during the day. Algae are also able to use reducing equivalents from the photosynthetic electron transport chain under some conditions to directly reduce hydrogenases at the level of ferredoxin without the input of ATP, a pathway that is theoretically regarded as the most efficient biological means to transform the energy in sunlight to H_2 for biotechnological applications [19]–[23]. To date, only FeFe-hydrogenases have been unambiguously identified in algae, with organisms such as Chlamydomonas reinhardtii encoding truncated enzymes with only the catalytic H-cluster; a 4Fe4S cluster linked via a bridging cysteine to a two Fe center coordinated by CN^-/CO ligands and a bridging dithiolate. In contrast, Chlorella variabilis NC64A encodes two hydrogenase enzymes with both the H-cluster catalytic domain and an F-cluster domain that coordinates additional FeS clusters that putatively function in electron transfer [18],[19], [24]–[26].

Despite widespread interest in algal H_2 production, contemporary research is focused almost exclusively on freshwater species of Chlamydomonas, Scenedesmus, and Chlorella, with C. reinhardtii being the model system for the vast majority of algal hydrogenase research [27],[28]. Significant advances in H_2 photoproduction from C. reinhardtii have recently been reported [29]–[33]; whereas relatively few studies have examined H_2 production from marine or halophilic algae [34]–[36].

Halophilic algae offer several advantages for large-scale algal H_2 production. First, the use of halophilic algae will enable H_2 production in readily available salt water, thereby minimizing the potential use of limited fresh water resources. Secondly, gas solubility is reduced in aquatic saline systems [37], which is potentially

advantageous because the levels of soluble O_2, a potent inhibitor of most FeFe-hydrogenases [38], are diminished in salt water relative to fresh water. Hydrogen is also less soluble in saline systems and therefore more easily removed. Lastly, ATP hydrolysis is increased in saline media to maintain proper cellular ion gradients. Hydrogenase activity is an ATP neutral process, and photosynthetic electron transport has been shown to decline as photosynthetic ATP levels rise during H_2 photoproduction in some freshwater algae [39], [23]. Salt stress represents a potential mechanism to alleviate ATP accumulation, which can inhibit the metabolic pathways supplying reductant to hydrogenase.

To further expand our understanding of H_2 metabolism in extreme environments, and to potentially discover novel organisms with enzymes that have superior biotechnological attributes, we initiated an effort to isolate hydrogen-producing organisms from hypersaline ecosystems. Although salt water systems are ubiquitous around the world, relatively little is known regarding H_2 metabolism in saline systems, or whether unique enzyme features are necessary for activity in halophiles [40], [41].

METHODS

Algal Strains and Growth Conditions

Halophilic algae from the genus Tetraselmis (Chlorophyceae) were isolated from water samples collected at (a) a roadside pool bordering the Great Salt Lake (GSL), Utah, USA, with a salinity of 6.0% w/v (Tetraselmis GSL1) and (b) a hypersaline pond with a conductivity reading of 660 mS/cm near Quemado, New Mexico, USA [42] (Tetraselmis QNM1). GSL samples were taken in conjunction with the Great Salt Lake Institute, which holds a permit for sampling on Utah state lands, granted by the Utah Department of Natural Resources, Division of Forestry, Fire and State Lands. Water sampling at the New Mexico site was conducted on public lands administered by the Bureau of Land Management, and did not require a permit nor involve endangered or protected species. Both Tetraselmis isolates have been submitted to the University of Texas (UTEX) algal culture collection.

Isolates were routinely cultured in f/2 medium amended with Booth Bay sea water (National Center for Marine Algae and Microbiota, NCMA), at pH 8.0 [43] and 3.5% of salinity. Where indicated, salinities were increased to 5.5% or 7.0% w/v with NaCl addition. Cultures were maintained at 29°C, without agitation at a constant illumination of ~30 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) by cool-white fluorescent lights. Cell counts were assessed using a Z2 Coulter cell and particle counter (Beckman-Coulter).

C. reinhardtii strain CC124 (nit^-, mt^-) was obtained from the Chlamydomonas Genetic Center (http://www.chlamy.org/) and grown in tris–acetate–phosphate (TAP) medium pH 7.2 [44], and shaken at 120 rpm under constant fluorescent irradiance (80 μmol m^{-2} s^{-1} of PAR by GE Ecolux 6500K T5 bulbs). Cells were harvested during mid-logarithmic growth (16–20 μg Chl/ml) for measuring in vitro hydrogenase activities and fermentative H_2 production.

Chlorophyll and Total Protein Determination

Total chlorophyll was determined spectrophotometrically by extraction in 100% methanol for the Tetraselmis strains and 95% ethanol for C. reinhardtii [44]. Tetraselmis cells were washed with 2 volumes of MilliQ water to remove salt prior to pigment extraction. Total chlorophyll was selected as the standard in normalizing hydrogenase activity with the light-absorbing capacity of isolates.

Total protein content was analyzed using a Modified Lowry Protein Assay (Pierce) according to the manufacture's instructions. Cell pellets were washed with 2 volumes of MilliQ water and then solubilized in 0.5% SDS. Total protein was quantified using BSA solubilized in 0.5% SDS to generate a standard curve.

Anaerobic Induction

For H_2-photoproduction measurements, 75 ml of mid-log phase cell cultures were grown at the indicated NaCl levels (3.5%, 5.5% and 7.0% w/v), concentrated by centrifugation at 3716 × g for 15 min at 25°C, resuspended in 1 ml of f/2 medium (at the same salinity used for culturing) and transferred to 16-ml glass serum vials covered with aluminum foil to exclude light. For fermentative metabolite analyses,

dark H_2-production and CO_2-evolution measurements, 50 ml of mid-log phase cell cultures were harvested, resuspended in 1 ml of fresh medium, and transferred to 16-ml glass serum vials covered with aluminum foil. For in vitro hydrogenase activity assays (see below), 1 ml of mid-log liquid cell cultures was directly transferred to anaerobic vials. For C. reinhardtii, in vitro hydrogenase activity and fermentative H_2 production, 5 ml of cells from mid-log cultures were harvested by centrifugation (3190 × g for 10 min) and resuspended in 1 ml of anaerobic induction buffer (AIB; 50 mM potassium phosphate buffer, pH 7.2, 3 mM $MgCl_2$), then transferred to 13-ml glass serum vials covered with aluminum foil. All vials were sealed with septa, purged for 30 min with ultra-high purity argon and kept sealed in the dark for 4 h or 24 h, as indicated.

H_2-Photoproduction Measurements

Maximum in vivo H_2-photoproduction rates were determined using a custom-built Ag/AgCl polarographic electrode system (ALGI). H_2- and O_2-photoproduction rates were measured simultaneously with two YSI 5331A electrodes (YSI Incorporated, Yellow Springs, OH, USA), poised at +/− 0.6 V, in a water-jacketed (25°C) assay chamber. O_2 and H_2 electrodes were calibrated between each measurement using f/2 medium (at the respective salinity of tested cells) saturated by atmospheric O_2 or by ultra-high purity 5.3% H_2 (Ar balance), respectively. Pure Ar purging was used to determine electrode baselines and to sparge the assay chamber containing 0.8 ml of f/2 medium of O_2. 0.2 ml of 4 h and 24 h anaerobically induced cells were then introduced into deoxygenated buffer in the sample chamber. After a 30 s dark acclimation, cells were illuminated for 30 s, using saturating LED (Luxeon III Star, Lumileds) irradiance of 2000 µmol photons m^{-2} s^{-1}. H_2-photoproduction rates were calculated from the initial slope during the first 10 s of illumination from the H_2-dependent current increase.

In Vitro Hydrogenase Activity

In vitro Tetraselmis GSL1 and QNM1 H_2-production activity assays were performed by transferring 1 ml of 2X methyl viologen (MV) buffered solution (10 mM MV, 50 mM potassium phosphate, pH 6.9, and 0.2% triton X-100) and 0.2 ml of reduced sodium dithionite