Algal Research 16 (2016) 36–45

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Rapid budding EMS mutants of *Synechocystis* PCC 6803 producing carbohydrate or lipid enriched biomass

Vikas Kumar Patel [a,b], Deepamala Maji [a], Shiv Shanker Pandey [a], Prasant Kumar Rout [c], Shanthy Sundaram [b,*,1], Alok Kalra [a,*,1]

[a] Microbial Technology Department, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow 226015, India
[b] Centre of Biotechnology, Nehru Science Centre, University of Allahabad, Allahabad 211002, India
[c] Process Chemistry and Technology Department, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow 226015, India

ARTICLE INFO

Article history:
Received 5 August 2015
Received in revised form 15 December 2015
Accepted 28 February 2016
Available online xxxx

Keywords:
Synechocystis PCC 6803
EMS mutants
Lipids
Carbohydrate
Chlorophyll a fluorescence
Biomass

ABSTRACT

The genome of wild-type (WT) *Synechocystis* PCC 6803 was altered by random mutagenesis using ethyl methane sulphonate and mutated cells exhibiting rapid growth, higher carbohydrate, and lipid yields were selected. Among these, eight rapid growing, dark green mutant strains viz. D1, D5, D6, D7, D8, D9, D10, and D14 were selected and compared for growth rates, dried biomass, total lipids, carbohydrate yields and chlorophyll a fluorescence with WT *Synechocystis*. Finally, two fast-growing mutants, D1 and D8, based on the maximum carbohydrate or lipid yields respectively, were selected. They could produce 3.6 and 4.2 fold more cells than WT *Synechocystis*, which resulted in higher biomass and lipid yields at the end of fermentation. Mutant D1 produced 46%, and 64% increased carbohydrate and lipids respectively while D8 produced 82% more lipids over WT *Synechocystis*. These strains hold potential to produce higher amounts of lipids or carbohydrates, and could be important bio-resources for the biofuel industries.

1. Introduction

Cyanobacteria are the oldest known photosynthetic, oxygen evolving, prokaryotes. Besides enhancing the oxygen concentration in the atmosphere [1], they are also considered important for their fundamental contribution to the global carbon cycle as well as in continuously increasing atmospheric CO_2 mitigation [2–3]. The autotrophic organisms carry out the initial energy storage reactions of photosynthesis, resulting in accumulation of primary products. Due to lipid rich biomass with significant amounts of essential fatty acids such as linoleic acid, α-linolenic acid and γ-linolenic acids, fresh and marine water forms of cyanobacteria are being considered as emerging biofuel agents [4]. With day to day increasing instability of fossil fuel, energy crisis, the realism of oil prices and a large increase in the greenhouse gases in the near future has invigorated a growing interest towards cyanobacterial as well as algal biofuels [5].

Presently, most of the biofuels use agricultural crops and their residues, which raise concerns about the food security with shrinking cultivable lands. As a third generation biofuel agents, unicellular eukaryotic algae, and prokaryotic cyanobacteria provide a useful link between carbohydrate assimilation and biofuel feedstock synthesis, chiefly lipids and carbohydrates. It is well-known that the yield of lipids and carbohydrates are several folds greater in cyanobacteria than competing first generation oil seed crops and second generation ligno-cellulosic plant cell wall materials [6]. Cyanobacteria include some fast growing species with higher carbohydrate or lipid contents in their biomass as compared to the traditional energy crops [7]. Unlike crops, they do not need fertile land and can reach high densities in much shorter time by efficiently utilizing solar energy, CO_2 and water containing small amounts of inorganic nutrients [8]. The economical production of large quantities of carbohydrate and lipid rich biomass is highly important for the commercialization of sustainable cyanobacteria based biofuels. Researchers throughout the world are applying the tools of genetic engineering, mutagenesis and bioprocess fermentation to further improve the productivities of triglycerides (TAGs) and carbohydrate in eukaryotic algae and prokaryotic cyanobacteria. Significant successes in the genetic manipulation using either mutagenesis or genetic engineering have recently been achieved with some algal and cyanobacterial systems [9]. These efforts are still ongoing globally to further manipulate central carbon mitigating metabolism in these organisms to sort out the issues associated with the first, second and third-generation biofuel technologies for developing the sustainable biofuels [9–11].

Although, being a photosynthetic model organism; *Synechocystis* is being explored for various biofuels using the genetic manipulation tools [12–13], efforts have been made to generate the temperature

* Corresponding authors.
E-mail addresses: Shanthy_s@rediffmail.com (S. Sundaram), alok.kalra@yahoo.com (A. Kalra).
[1] Both corresponding authors contributed equally.

http://dx.doi.org/10.1016/j.algal.2016.02.029
2211-9264/

tolerant chemical mutants in cyanobacteria; even *Synechocystis* PCC 6803 has been mutagenized with methyl methane sulphonate (MMS) to obtain the temperature tolerant mutants [14]. However, no or limited efforts towards producing mutants of *Synechocystis* PCC 6803 for better growth and biomass, lipid or carbohydrates production have been made.

Although, *Synechocystis* is a fast growing organism and can produce lipids and carbohydrates in substantial good quantities, however, to use it as a commercial biofuel agent, further improvements in its growth and contents of carbohydrate and lipids are essential. Using chemical mutants are considered safer than genetically manipulated strains [15], as genetically manipulated strains, when cultivated in the open systems or fields, may become a source of transfer of the antibiotic resistance to their relative microbes. Keeping this in mind, chemically induced mutagenesis for the production of rapid growing, carbohydrate or lipid-enriched *Synechocystis* strains was attempted, as it provides better acceptability with minimal environmental concerns over genetic engineering methods.

The main objective of this study was to develop some unicellular cyanobacterial strains that have the potential to grow rapidly, producing higher biomass, and enhanced yields of carbohydrate and lipids by employing chemical mutagenesis and involving an indigenous strain of *Synechocystis* PCC 6803; having the capability to grow both under photoautotrophic and heterotrophic conditions. This work is a first attempt to generate the rapid growing, carbohydrate or lipid enriched strains of *Synechocystis* PCC 6803 by applying EMS mutagenesis.

2. Materials and methods

2.1. Growth conditions and culture maintenance

The WT *Synechocystis* PCC 6803 was obtained from National Phytron Facility Centre, IARI, Pusa, New Delhi, India. Throughout the experiments, WT and mutant *Synechocystis* strains were cultivated in 100 mL of $BG11^+$ media (having L^{-1} composition as 1.5 g, $MgSO_4 \cdot 7\ H_2O$; 0.036 g, $CaCl_2 \cdot 2H_2O$; 0.006 g, citric acid; 0.06 g, ferric ammonium citrate; 0.001 g, Na_2EDTA; 0.02 g, Na_2CO_3; and 1 mL of microelements stock solution L^{-1} composition as 8.82 g, $ZnSO_4 \cdot 7H_2O$; 1.44 g, $MnCl_2 \cdot 4H_2O$; 0.71 g, MoO_3; 1.57 g, $CuSO_4 \cdot 5H_2O$; 0.49 g, Co $(NO_3)_2 \cdot 6H_2O$). The media was buffered at pH 7.8 with 10 mM HEPES [16–17]. The cultures were incubated at 30 ± 1 °C in a thermostatically controlled chamber, illuminated with cool fluorescence lamps (Phillips 40 W, cool daylight 6500 K) at an intensity of 46.25 μmol m^{-2} s^{-1} photons in a 14:10 h light–dark regime. To maintain the strains on agar plates, the $BG11^+$ media was supplemented with 1.8% (*w*/*v*) agar and 1 mM sodium thiosulphate.

2.2. Mutagenesis and screening of rapid growing mutants

WT culture (5 mL) of *Synechocystis* PCC 6803 in its exponential growth phase (OD_{730} = 1.0) was harvested by centrifugation at 1250*g* for 5 min. Cells were washed with sterile $BG11^+$ media, resuspended into 500 μL of 0.1 M phosphate buffer (pH 7.0), and exposed to the five different concentrations (0.23 M, 0.47 M, 0.70 M, 0.94 M, 1.18 M) of mutagen EMS (Ethyl Methane Sulphonate, procured from SRL Sisco Research Laboratory Pvt.) for 1 h in the dark at room temperature [18]. The activity of EMS was inactivated by adding the freshly made sterile 10% (*w*/*v*) sodium thiosulphate for 10 min. Cells were washed with 1 mL phosphate buffer (0.1 M), centrifuged at 1250*g* for 5 min, and resuspended the pellet in 1 mL of $BG11^+$ media and kept for 24 h in the dark. The mortality rate was estimated by counting the motile cells under 100× oil immersion objective. Suitable dilutions of survivors were made with the $BG11^+$ medium. Aliquots of the mutagenized cultures were spread to $BG11^+$ media supplemented with 1.8% agar in petri dishes and incubated for 2 to 4 weeks. Conversion of EMS-treated cells into the colonies was regularly monitored. Mutant colonies were screened visually on the basis of their morphological appearance (dark green color and altered size). Fourteen dark green and fast growing colonies named as D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, D11, D12, D13 and D14 were isolated from the plated cells; treated with 1.18 M EMS. Subsequently, these fast growing colonies were grown again and again on $BG11^+$ petri plates up to 12 generations under the same growth conditions. The subsequent colonies from the mutants D2, D3, D4, D11 and D13 were lost during the successive generations. Only colonies from D1, D5, D6, D7, D8, D9, D10 and D14 could survive up to 12 generations and, therefore, were selected for further experiments. The colonies from the different surviving mutants were transferred in 10 mL liquid $BG11^+$ medium in test tubes. Furthermore, during the start of each experiment; an equal number of cells of WT and mutants (standardized on the basis of a hemocytometer and corresponding O.D.) from the exponentially grown cultures were inoculated in an equal volume of $BG11^+$ liquid media. While at the end of experiments, either equal volume (100 mL) or equal biomass were considered for various analysis.

2.3. Growth kinetics of wild-type and mutant Synechocystis PCC 6803 strains

The initial cell density of 4×10^7 WT *Synechocystis* PCC 6803 and its mutant strains (mL^{-1}) from exponentially grown cultures were inoculated in 100 mL of $BG11^+$ media for studying the growth kinetics. Cyanobacteria cell density was determined after an interval of every 24 h by measuring the absorbance at OD_{730} using Specord 50 version 2.0 E, a UV–VIS spectrophotometer and cell counting by a hemocytometer. The population density of all the mutants was standardized and measured considering that 0.5 units OD at 730 nm wavelength is equal to 1.21×10^8, 1.14×10^8, 1.14×10^8, 1.14×10^8, 1.41×10^8, 1.15×10^8, 1.14×10^8, 1.14×10^8 cells mL^{-1} for D1, D5, D6, D7, D8, D9, D10 and D14 respectively. The cell density of WT was considered as 1.0×10^8 cells mL^{-1} at OD_{730}. Therefore, for all subsequent experiments, the OD values were extrapolated into population densities for both WT and mutants. Same parameters were considered throughout the experiments. The number of generations 'n', mean generation time 'g' (d^{-1}) and specific growth rate 'μ' (d^{-1}) in logarithmic growth phase were calculated according to the following equations [19].

$$n = \frac{\log N - \log N_0}{\log 2} \tag{1}$$

$$g = \frac{\text{Total time in log phase}}{\text{Number of generations}} \tag{2}$$

where; N_t and N_0 are the final and initial cyanobacterial cell numbers (cells mL^{-1}), 'n' is the number of generations, and 'g' is the mean generation time. The specific growth rate 'μ' (d^{-1}) in logarithmic growth phase was calculated for these cyanobacteria according to the equation.

$$\mu = \ln\left(\frac{N_t}{N_0}\right)/t \tag{3}$$

where; N_0 and N_t represent the cell density values at t = 0 and 't' h after the start of the growth, respectively. The various strains were sampled on the 14th day of cultivation for the quantification of biomass. The calculation of growth capacity of mutants was as follows.

$$\text{Growth capacity}(\%) = \frac{\text{Biomass productivity of mutant strain} \times 100}{\text{Biomass productivity of WT } \textit{Synechocystis}} \tag{4}$$

2.4. Surface to volume ratios

The surface area, volume and surface area to volume ratios of WT *Synechocystis* PCC 6803 and different mutated cells were measured, on the basis of the diameter of different cells as observed in the micrographs