Algal Research 27 (2017) 140–151

Contents lists available at ScienceDirect

Algal Research

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

A comprehensive assessment of the biosynthetic pathways of ascorbate, α-tocopherol and free amino acids in *Euglena gracilis* var. *saccharophila*

Mafruha T. Hasan[a,b,c], Angela Sun[a,b,c], Mehdi Mirzaei[a,b,c,d,e], Junior Te'o[b,f], Graham Hobba[b,g], Anwar Sunna[a,b,c], Helena Nevalainen[a,b,c,*]

[a] Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia
[b] ARC-MQ Industrial Transformation Training Centre (ITTC) for Molecular Technology in the Food Industry, Sydney, Australia
[c] Biomolecular Discovery and Design Research Centre, Macquarie University, Sydney, Australia
[d] Faculty of Medicine and Health Sciences, Macquarie University, Sydney, Australia
[e] Australian Proteome Analysis Facility, Macquarie University, Sydney, Australia
[f] School of Earth, Environmental and Biological Sciences, Queensland University of Technology, Brisbane, Australia
[g] Agritechnology Pty Ltd, Borenore, Australia

ARTICLE INFO

Keywords:
Euglena gracilis var. *saccharophila*
Proteomics
Biosynthetic pathways
Ascorbate
α-Tocopherol
Free amino acid biosynthesis

ABSTRACT

Euglena gracilis produces several important health-enhancing metabolites including ascorbate, α-tocopherol and free amino acids (faa). The yield of metabolites is dependent on the strain of *E. gracilis* and the metabolic growth condition. Here we investigated the effects of photoautotrophic (PT), mixotrophic (MT) and heterotrophic (HT) cultivation on the synthesis of ascorbate, α-tocopherol and faa in *E. gracilis* var. *saccharophila*, using label-free shotgun proteomics, and metabolite analysis using colourimetric assay, high-performance and ultra-performance liquid chromatography (HPLC/UPLC). PT cultivation resulted in the production of more antioxidants (up to 4.13 mg g^{-1} ascorbate and 2.52 mg g^{-1} α-tocopherol) than the MT and HT growth conditions (up to 0.97 and 0.50 mg g^{-1} ascorbate, and 1.40 and 0.21 mg g^{-1} α-tocopherol, respectively). The relative abundance of several faa varied between mid-log and initial stationary growth phases, but the total amount of faa remained about the same, with arginine as the most abundant amino acid. Proteomic analysis revealed a total of 3843 non-redundant proteins in *E. gracilis* var. *saccharophila*, of which 1890 were common among all cultivations. Gene ontology annotations suggested derivatisation of metabolic pathways from different organisms, such as lysine biosynthesis from fungi and serine biosynthesis from plants, while a few pathways were unique to *Euglena*, such as those of ascorbate and arginine. Some enzymes exhibited several isoforms that were influenced by the metabolic growth condition. For example, one of the isozymes of threonine aldolase was expressed in HT/MT cultures only, and one of the isozymes of phosphoglycerate dehydrogenase was expressed in PT cultures only. This is the first proteomic study of *E. gracilis* var. *saccharophila*, which provides a mechanistic insight into the biosynthetic pathway dynamics of primary metabolites (antioxidants and faa). This new information can serve as a framework for further development of *Euglena* as a producer of nutraceuticals.

1. Introduction

Microalgae have recently attracted interest as a platform for several industrial applications owing to their metabolic versatility. *Euglena gracilis* is a freshwater microalga that can be employed for bioremediation of heavy metals and toxic substances from polluted water [1,2], CO_2 sequestration [3], toxicity testing [4], biodiesel and biogas production [5,6], vitamin B_{12} assays [7], photo-, gravi- and polaro-taxis studies [8,9], nanoparticle and nano-fiber synthesis [10,11], and nutraceutical biosynthesis [12]. This wide range of uses can be attributed to the remarkably flexible metabolism of *E. gracilis*, acquired from a complex evolutionary history, with genes assimilated from proteobacteria, red algae and photosynthetic organisms [13]. *E. gracilis* thus has a vast armoury of genes that it can access to adapt to different environmental conditions.

E. gracilis naturally produces several compounds that have health-enhancing properties, including antioxidants that can remediate oxidative stress. Oxidative stress inside cells has been reported to have a strong correlation with numerous diseases, including cancer [14], cardiovascular diseases [15] and Alzheimer's disease [16]. *E. gracilis* also

* Corresponding author at: Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia.
E-mail address: helena.nevalainen@mq.edu.au (H. Nevalainen).

http://dx.doi.org/10.1016/j.algal.2017.08.029
Received 17 April 2017; Received in revised form 18 August 2017; Accepted 30 August 2017

synthesises all twenty of the protein-building amino acids, eight of which are essential in the human diet and four required by infants and young children [12]. The amount of health-enhancing organic compounds produced in *E. gracilis* depends largely on the strain and cultivation method used. Metabolic adaptability allows this alga to grow under photoautotrophic (PT) cultivation using inorganic CO_2 for photosynthesis, heterotrophic (HT) cultivation using organic carbon sources from its environment, as well as mixotrophic (MT) cultivation using both CO_2 and organic compounds. Several studies have been carried out to identify metabolites produced by *E. gracilis* under the influence of light and dark, and various carbon sources [12,17,18]. However, the effect of these cultivation conditions on the enzymes and pathways that produce the metabolites has not been examined in *E. gracilis* var. *saccharophila* before. The most common strain of *E. gracilis* used for metabolite studies is *E. gracilis* Z, which favours photosynthetic growth, although it can grow heterotrophically as well. Little is known about its sugar-loving counterpart, *E. gracilis* var. *saccharophila* and its metabolite production capabilities.

The initial features of the *E. gracilis* genome have recently been described [19]. However, the genome has not been fully annotated or made publicly available yet. The recent publication of the transcriptome has revealed insight into its exhaustive metabolic competency, with 32,128 predicted proteins [20]. A comprehensive translated proteome of *E. gracilis* will be difficult to compile as some proteins are only expressed when certain conditions are met. Moreover, the potentially expressed proteins described in the transcriptomic paper may differ from the actual proteins that are translated since *E. gracilis* extensively alters mRNA sequences prior to translation [20]. Nevertheless, in this study we aimed to identify the enzymes and pathways that *E. gracilis* var. *saccharophila* utilises to produce ascorbate, α-tocopherol and the twenty protein-building amino acids under PT, HT and MT cultivations. Label-free shotgun proteomics was used to find enzymes expressed at the proteomic level, and this was compared to data from metabolite analysis. We believe that this work will help to elucidate those transcripts that are preferentially translated in this strain, under PT, MT and HT cultivation respectively, from the wide range of predicted protein isoforms. This will in turn provide a valuable opportunity to explore metabolic networks in *E. gracilis*, which can be used for functional analysis and overexpression of the metabolites of interest.

2. Materials and methods

2.1. Growth medium

E. gracilis var. *saccharophila* (UTEX 752) was obtained from the University of Texas Culture Collection, USA. The strain was maintained as a pure axenic culture using modified Hutner medium, as the basal medium (pH 3.5), supplemented with vitamins B_1 and B_{12}, as described previously [12], with the addition of 10 g/l yeast extract (YE). For PT cultures (Section 2.2 below), the basal medium was prepared by dissolving all minerals and bases [12] in 1 l of MilliQ water, whereas for MT and HT cultures (Section 2.2 below), the same quantities of minerals and bases were dissolved in 925 ml of MilliQ water. The autoclaved basal medium for PT cultures was used directly for inoculation, whereas for the MT and HT cultures, the autoclaved basal medium was supplemented with 75 ml of separately autoclaved 20% D-(+)-glucose before inoculation, so that the final concentration of glucose in the medium was 1.5%. The glucose for the MT and HT cultures, and the vitamins for all cultures were added only after cooling the medium to room temperature, before inoculation. All chemicals and reagents were purchased from Sigma Aldrich, Australia unless stated otherwise.

2.2. Culture conditions

Cultivation was carried out under three different nutritional modes: PT (photoautotrophic), MT (mixotrophic) and HT (heterotrophic). The difference between the cultures was the source of carbon. PT cultures were able to utilise mainly atmospheric CO_2 via photosynthesis, with some carbon from the amino acids present in YE. The HT cultures (in the dark) utilised an organic carbon source (glucose) with YE. MT cultures were able to utilise both atmospheric CO_2 and glucose from the medium, along with YE.

Each set of cultivations was performed with three biological replicates. The PT cultures were grown in basal medium containing no glucose and were exposed to white light illumination (2000 lx) for a photoperiod of 14 h light/10 h darkness. The MT cultures were grown in the same lighting condition as PT, but the basal medium was supplemented with glucose. The HT cultures were grown in the same medium as MT cultures, but in complete darkness. Light-adapted and dark-adapted stock cultures, maintained under the conditions mentioned above, for over 2 years, were used to inoculate the PT and MT cultures, and HT cultures respectively. All stock cultures were starved (basal medium not replenished) for 3 days prior to inoculation, and no glucose was added to the stock cultures for 10 days prior to inoculation. For each culture, an initial concentration of 1.5×10^8 cells of the respective stock was inoculated in 150 ml of medium in 250 ml Erlenmeyer flasks. All cultures were incubated at 23 °C with orbital shaking at 150 rpm.

2.3. Determination of dry mass

Every 24 h, 5 ml of cells were harvested by centrifugation at 5000*g* for 10 min and the supernatant was discarded. The pellet was dried in a pre-weighed aluminium evaporating dish at 70 °C for 48 h. The dish was cooled to room temperature in a desiccator for 10 min before weighing.

2.4. Determination of ascorbate

Ascorbate was determined every 24 h, for 72 h, from inoculation to the start of the stationary phase. The cells were harvested, washed twice with 10 ml of cold Milli-Q water, and resuspended in 3 ml of cold lysis buffer. The lysis buffer was prepared by dissolving one PBS tablet (MP Biomedicals, Australia) in 100 ml of Milli-Q water, supplemented with 0.04% (v/v) β-mercaptoethanol and protease inhibitor (2 tablets of cOmplete™ Protease Inhibitor Cocktail, Roche) before use. The samples were lysed by French press treatment (Thermo Spectronic French Pressure Cell Press, MA, USA) at 15,000 psi. The lysate was centrifuged at 2000*g* for 5 min. One hundred μl of the lysate supernatant was deproteinised by ultrafiltration through a 10 kDa nominal MWCO centrifugal filter (Amicon® Ultra Centrifugal Filters, Merck Millipore, Australia) by centrifuging at 14,000*g* for 1 h. The remaining lysate supernatant was used for free amino acid determination (Section 2.6) and protein extraction (Section 2.7) described below, for the respective days. Ascorbate from the deproteinised flow-through was then measured using the Ascorbic Acid Assay Kit (MAK074, Sigma Aldrich, Australia) as per the manufacturer's protocol.

2.5. Determination of α-tocopherol

Alpha tocopherol was determined at the same time points as ascorbate. Extraction was carried out as previously described [12]. An Agilent 1260 Infinity Semi-Preparative High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) system (Agilent Technologies, Australia) was used to determine α-tocopherol. A Symmetry C18 150 mm × 3.5 μm reverse phase HPLC column (i.d. 2.1 mm, Waters, Australia), with an injection volume of 10 μl and a flow rate of 0.5 ml min^{-1} was employed at room temperature. An isocratic method was used, eluting the components with a methanol:acetonitrile (50:50 v/v) mobile phase, and α-tocopherol was detected by UV absorbance at 294 nm. Quantitation was carried out using a standard calibration