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Full Length Article

Evaluation of the ethanol tolerance for wild and mutant *Synechocystis* strains by flow cytometry

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ABSTRACT

Flow cytometry was used to evaluate the effect of initial ethanol concentrations on cyanobacterial strains of *Synechocystis* PCC 6803 [wild-type (WT), and ethanol producing recombinants (UL 004 and UL 030)] in batch cultures. Ethanol recombinants, containing one or two metabolically engineered cassettes, were designed towards the development of an economically competitive process for the direct production of bioethanol from microalgae through an exclusive autotrophic route.

It can be concluded that the recombinant *Synechocystis* UL 030 containing two copies of the genes per genome was the most tolerant to ethanol. Nevertheless, to implement a production process using recombinant strains, the bioethanol produced will be required to be continuously extracted from the culture media via a membrane-based technological process for example to prevent detrimental effects on the biomass. The results presented here are of significance in defining the maximum threshold for bulk ethanol concentration in production media.

1. Introduction

The global energy crisis and the international political pressure to reduce greenhouse gases (GHG) have driven the search for renewable energy alternatives to replace traditional energy sources [1]. The production of bioethanol has received increasing attention recently, as it is one major type of biofuel that can be blended with gasoline for use in current internal combustion engines powertrains.

Currently, most bioethanol is obtained by fermentation of starch-based cereals and sugarcane. However, biofuels produced from food crops are in competition with the food and animal feed industries, increasing food prices and rising strong opposition in the EU and globally [2]. On the other hand, forest and agricultural lignocellulosic residual materials display a severe recalcitrance towards fractionation for sugars production, and the inability of microorganisms to efficiently ferment lignocellulosic hydrolysates still reduce the bioethanol process yield from these plentiful materials [3].

Therefore, new (bio)technical routes for the production of bioethanol that are not lignocellulosic biomass based but directly photosynthesis-derived are the subject of intense research [4–6]. Cyanobacteria are prokaryotes which exhibit diversity in metabolism, structure, morphology and habitat and perform photosynthesis similar to that performed by higher plants [7]. In addition, as cyanobacteria have simple growth requirements, grow to high densities, and use light, carbon dioxide, and other inorganic nutrients efficiently, they could be attractive hosts for production of valuable organic products [8].

Synechocystis sp. strain PCC 6803 (hereafter *Synechocystis*) is a model cyanobacterium for genetic manipulation and has been already widely used as a cell factory to produce a range of biotechnological products such as ethanol, fatty acids, biopolymers and sugars [6]. Within the FP7 DEMA project (Direct Ethanol from MicroAlgae) the utility of this model cyanobacterium to produce ethanol at a low cost was investigated (http://www.dema-etoh.eu/en/). A non-native ethanol biosynthesis pathway has been integrated into *Synechocystis* and a library of ethanol-producing recombinants has been generated and characterized, both genetically and phenotypically, including strains UL 004 (a single copy strain) and UL 030 (a double copy strain), which, under laboratory conditions, have been producing encouraging levels of ethanol [9].

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Cyanobacteria and the model organism *Synechocystis* are known to be sensitive to ethanol [10], which may limit efforts to increase ethanol production levels in these microorganisms thus examination of production levels and tolerance are key determinants of the utility of such a cell factory.

Flow cytometry has been extensively used in microalgae stress studies, being considered an useful technique to detect microalgal cell stress response to environmental stress [11–16].

The detection of cellular esterase activity (which concerns common enzymes in viable cells of both plants and animals) by cytometry, can be an indicator of the cell viability as well as of metabolic activity [16]. In addition, it is an effective bioassay since it can offer acute and sub-lethal endpoints and help to identify inhibition mechanisms [12,14,15].

Ethanol toxicity, on the other hand, is generally attributed to the preferential partition of ethanol in the hydrophobic environment of microbial membrane lipid bilayers, resulting in the perturbations of the membrane structure. Therefore, as the plasma membrane protects the cell from its external environment, membrane integrity is considered an important cell viability indicator when studying the effect of solvents such as ethanol on microbial cells [17].

Franklin et al. [12] developed a rapid enzyme inhibition detection method based on Fluorescein diacetate/Propidium iodide (FDA/PI) staining for microalgae using flow cytometry, which was successfully utilized by Xi et al. [14] to evaluate the viability of the cyanobacterium *Microcystis aeruginosa*.

To understand the mechanism(s) of ethanol tolerance in *Synechocystis* and two recombinants with improved ethanol production capacity (UL 004 and UL 030, engineered at the University of Limerick), the present work evaluated their stress response when grown in batch cultivation in the presence of increasing initial ethanol concentrations. Flow cytometry was then used to monitor the cellular enzymatic activity and membrane integrity during the growth assays.

The results reported increase our understanding of ethanol tolerance of *Synechocystis* cells (wild-type and recombinant strains), which may allow construction of more robust ethanol producers cyanobacterial strains.

2. Materials and methods

2.1. Synechocystis *strains*

Synechocystis (kindly provided Prof. Klaas Hellingwerf, University of Amsterdam) was routinely maintained at 30 °C on BG-11 media supplemented with 10 mM TES-NaOH (pH 8.2), 20 mM glucose and 0.3% (w/v) sodium thiosulfate. *Synechocystis* UL 004 is an ethanol producing strain of PCC 6803 containing a single gene cassette which consists of the P_{psbAII} light inducible promoter from *Synechocystis* [18], the pyruvate decarboxylase, *pdc*, gene from *Zymomonas mobilis* (Zm*pdc*), the native *Synechocystis adhA* gene [19], and the kanamycin resistance gene from ICE R391 [20]. The construct also contains 500 bp at each end with homology to the PSBAII neutral site to allow homologous recombination into this neutral site [21]. For construction of UL 004 (Fig. 1) the single cassette strain, a biobrick cloning and construction approach was used [22,23]. The kanamycin and *adhA* genes were amplified from their respective genomes with primers as described (primers 1 to 4, Table 1) to allow a subsequent overlap PCR to link the kanamycin and *adhA* genes. pMOTA [5] was linearised and used as the source or the Zm*pdc* fused to the P_{psbAII} promoter and the 500 bp homologous regions to allow integration into the PSBA2 neutral site. Homologous recombination via the In-Fusion® HD cloning kit (Clontech Laboratories Inc. 2014, Takara Bio Company, Mountain view, California, USA) was used to recombine the overlaping PCR product [KanR/*adhA* and linearised pMOTA], this was cloned into pUC18 [24] again via In-Fusion® HD cloning resulting in the construction of the UL 004 plasmid. Verification of the UL 004 plasmid was carried out via PCR amplification of the construct (primers 15 and 66, Table 1) followed by sequencing. The *Synechocystis* homologous psbA2 site within the UL 004 plasmid was utilized to allow homologous integration of the UL 004 cassette into the psbA2 neutral site in *Synechocystis* [21]. Transformants of wild type *Synechocystis* [25] were sub-cultured in BG-11 media containing increasing concentrations of kanamycin [5–50 μg/ml] until full integration of the UL 004 cassette was verified. Verification of integration into the psbA2 neutral site was carried out with appropriate primers (primer 5 and primer 6, Table 1) that bound the flanking homologous insertion site within psbA2. Wild type *Synechocystis* amplified with these primers generated a PCR product approximately 1.2 kb in size, insertion of the UL 004 cassette resulted in the amplification of a ~4 kb PCR product. Strains, designated UL 004, were also assayed for ethanol production to determine functionality of the cassette.

A double cassette strain, UL 030, was then constructed in a similar manner to that reported by Gao et al. [4] (Fig. 1) by inserting a second cassette into UL 004. This second cassette was inserted into the *phaAB* genes, eliminating PHA accumulation and storage, and hence, facilitating more pyruvate availability for conversion to ethanol [4]. Initially the UL004 plasmid was modified to remove the kanamycin resistance determinant and replace it with zeocin resistance utilising similar homologous recombination techniques as described above and primers as per Table 1. The UL 004 plasmid was linearised with inverse PCR at regions flanking the kanamycin resistance gene, thereby linearising the plasmid while removing the kanamycin gene. The zeocin resistance gene was amplified from pcDNA3.1 (Invitrogen, ThermoFischer Scientific) with primers 9 and 10 (Table 1). Homologous recombination of the linearised UL 004 plasmid lacking the kanamycin determinant and amplified zeocin resistance gene resulted in the generation of UL004-zeocin, a zeocin resistance version of the UL 004 plasmid.

Similarly a second unique plasmid was generated containing the *phaAB* genes and flanking regions from *Synechocystis*. The

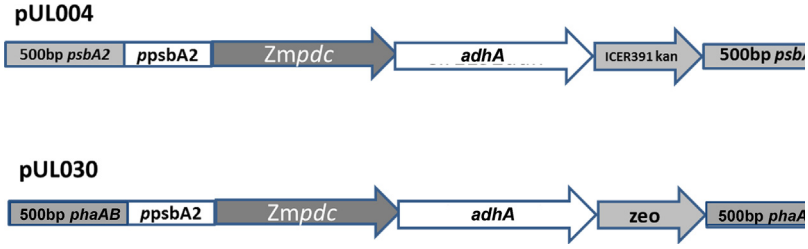

Fig. 1. Construction of UL 004 (a single cassette strain) and UL 030 (a double cassette strain).
The Genbank assession of the *Synechocystis adhA* was Gen Bank: AP012205.1 (nt 3,530,233 to 3,531,243), CDS: BAK51882.1; The GeneBank assession of the *Zymomonas pdc* was Gene Bank HM235920.1 (nt 493.2199) CDS: ADK13058.1.