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New constructs for ethanol production via cyanobacteria

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Abstract. Alternatives to fossil fuels must be developed due to several already known reasons. Bioethanol can be an attractive energy concept. Bioethanol gasoline hybrid fuel can be used by most internal combustion engines. First and second generation bioethanol production is already available – here agricultural crops or residues are utilised. There are controversial discussions about these bioethanol production methods – the food versus fuel debate, cost and energy efficiency. Alternative advanced bioethanol production must be established with competitive production costs. Photosynthetic prokaryotes like cyanobacteria are attractive organisms for this purpose – these prokaryotes are fast growing organisms and utilize solar energy and CO_2. But these prokaryotes must be genetically manipulated for ethanol production. In this study transformation was performed using homologous recombination to introduce the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase B (*adhB*) genes of *Zymomonas mobilis* into the photosynthetic prokaryote *Synechococcus elongatus* PCC 7942 genome. These cyanobacteria grow in fresh water and seawater or even in wastewater. Both genes were expressed under the control of the strong constitutive promoter of psbA1 gene (encoding photosystem II protein D1). Various cloning strategies were done. Each construct was transformed successful in *Synechococcus elongatus* PCC 7942 and the potential bioethanol production was determined with HPLC. Only one construct produces bioethanol at detectable level. Diverse reactors and scale up steps were done to increase the bioethanol production. Anyhow further cloning strategies must be implemented to improve the production rate to achieve an effective bioethanol production from *Synechococcus elongatus* PCC 7942.

Key words: bioethanol, third generation, cyanobacteria, genetic manipulation.

INTRODUCTION

For transportation bioethanol is a good possibility to replace fossil fuels, up to 10 percent gasoline can be replaced by bioethanol in each gasoline-powered vehicle without any modification. Bioethanol is actually produced from food crops (first generation bioethanol) or lignocellulose (second generation bioethanol) but also other routes for bioethanol production must be established to meet the demands. One route is the usage of photo-autotrophic microorganisms. The bioethanol production via photo-autotrophic microorganisms demands short fermentation times, high volumetric productivity, simple handling, low risk of contaminations and low operating cost. Microorganisms like cyanobacteria are in focus of the research – due their potential for photoautotrophic conversion of CO_2 into a broad range of industrially valuable compounds like isobutanol, isopropanol and also bioethanol (Ducat et al., 2010).

The first paper about the bioethanol production via *Synechococcus elongatus* was published in the year 1999 (Deng & Coleman, 1999). Several companies and working groups work on this issue using diverse cloning strategies and various cyanobacteria strains (reviewed in Dexter et al., 2015). The latest patent from the company Algenol reported a maximum rate of 0.552 g L^{-1} bioethanol after one day from the strain ABICyano1 (patent 2014178958). Anyhow for efficient bioethanol production via cyanobacteria higher ethanol concentration must be achieved in shorter time.

In this work several vectors were designed to achieve *Synechococcus elongatus* PCC 7942 strain that converts CO_2 to bioethanol. The *Synechococcus elongatus* PCC 7942 strain is a fresh-water cyanobacterium, obligate autotroph with a genome of approximately 2.7 Mb and two endogenous plasmids (Kaneko et al., 1996). It was the first cyanobacterium demonstrated to be reliably transformable by exogenously added DNA (Shestakov & Khyen, 1970). First the gene cassette pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase B (*adhB*) genes from *Z. mobilis* under the control of the strong constitutive promoter of psbA1 gene (encoding photosystem II protein D1) was used for the transformation of *Synechococcus elongatus* PCC 7942. The strong constitutive promoter of psbA1 gene was never used for ethanol production in *Synechococcus elongates*. In another study the psbAII promoter was used (Dexter & Fu, 2009) which differs according the publication of Mulo et al. (2009).

Furthermore diverse constructs from this cassette or adding of the *adhA* gene direct amplified from the genomic DNA of *Z. mobilis* were done to check a potential increase in the bioethanol production after successful transformation. Only the *Synechococcus elongatus* PCC 7942 transformed with the vector including the gene cassette exhibited ethanol production. The transformations of the other two vectors were successful but no bioethanol production was detectable. Further cloning must be done to achieve successful bioethanol production via cyanobacteria.

MATERIALS AND METHODS

Cloning vector and cloning stratagies

The vector pSyn_6 from the company Invitrogen (www.invitrogen.com) was used for each strategy. The vector contains among other important normal cloning sites two specific cloning sites – NS1 (neutral site 1) homologous recombination sites for the integration of the vector into the *Synechococcus elongatus* PCC 7942 genome and the strong constitutive promoter psbA1 gene (ending photosystem II protein D1) from *Synechococcus elongatus* PCC 7942 enabling the high level expression of the cloned gene. The construct pLOI295 was kindly provided by Lonnie O. Ingram (Department of Microbiology and Cell Science, University of Florida, Gainesville 32611). This construct contains the gene cassette encoding *adhB* and *pdc* gene from *Z. mobilis*. The construct P6C were produced using the construct pLOI295 as template via the PCR method. The other two constructs – were amplified via PCR from the construct pLOI295 or direct from the genomic *Z. mobilis* DNA as described in detail elsewhere (Pfannerer, 2014). Here each gene has its own promtor and terminator and was subcloned consecutively. The vector named P6PA included the gene *adhB* and *pdc* gene and the vector P6PAA contained an additionally gene, *adhA* see Fig. 1).

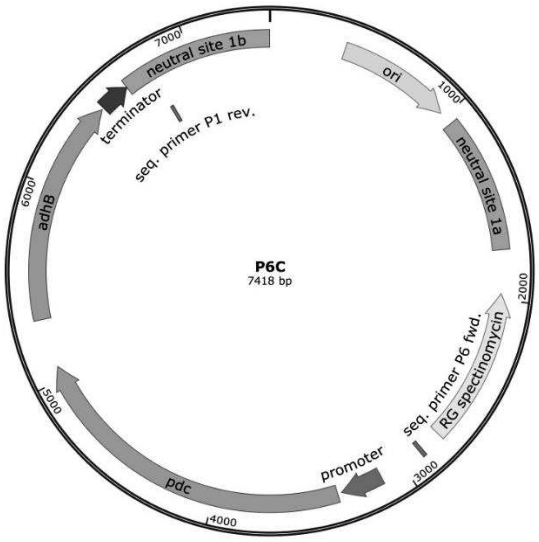

Figure 1. The map of the vector P6C – The vector with the name P6C included the original gene cassette encoding *adhB* and *pdc* gene.

PCR conditions and primers

The PCR was done with the forward and reverse primer (each 50 pM), 1 Unit Pfu Polymerase (company: Promega; www.promega.com) plus buffer, 200–500 ng template DNA, 10 mM dNTP Mix (company: Promega; www.promega.com), 3 µl DSMO (fill up to total volume of 50 µl with water). The sequences from forward and reverse primer from construct P6C were: 5´-GGA ATT CCA TAT GAG TTA TAC TGT CGG TAC C-3´and 5´-CCA ATG CAT TTA GAA AGC GCT CAG GAA GAG-3´. The PCR product was digested with NdeI and NsiI and ligated into NdeI and NsiI digested vector pSyn_6. Diverse subcloning steps were necessary for the construction of the other two constructs and are described in detail elsewhere (Pfannerer, 2014). Only the primers for the ampification of the *adhA* gene from the genomic DNA of *Z. mobilis* are mentioned: 5´-CAC CAT GAA AGC AGC CGT CAT AAC TAA A-3´and 5´-CTA GTG ATG GGT AAA ATC AAC AAC C-3´. Each construct was sequenced (company: VBC-Biotech; www. http://www.vbc-biotech.at/) to verify the correctness of the cloning. Transformation of *Synechococcus elongatus* was done according to the manufactory manual (www.thermofisher.com).

Ethanol measurements with HPLC

The potential ethanol production via cyanobacteria was measured with HPLC analyses. One milliliter of medium was transferred into a 1.7 mL centrifugation tube and centrifuged for 5 minutes at an rotational speed of 13,000 min^{-1} (centrifuge: Eppendorf 5417C with an angle rotor type FA 45-30-1). The clear supernatant was transferred into a 1.7 mL screw cap vial and stored at -18 °C until measurement. Ethanol concentrations were quantified by HPLC, using an Agilent Technologies 1200 Series equipped with a Varian Metacarb 87 H column (300 x 7.8 mm) at 65°C, H_2SO_4 (c = 5 mmol L^{-1}) eluent and an isocratic flow rate of 0.8 mL min^{-1} was used. The data acquisition was performed per refractive index detection and UV–detection at 210 nm.

For calibration the method of external standard was applied. Data analysis was performed per Agilent Chemstation 04.03 b.

Determination of the growth curve from *Synechococcus elongatus*

After transformation precultivation of *Synechococcus elongatus* positive clone were done in BG11 media after transformation at illuminance of 100 μE m^{-2} s^{-1}. The BG11 media persist of boric acid 0.00287%, manganese chloride tetrahydrate 0.00181%, zinc sulphate heptahydrate 0.00022%, sodium molybdate dehydrate 0.00039%, copper sulphate pentahydrate 0.00008%, sodium nitrate 0.15%, calcium chloride dehydrate 0.0027%, ferric ammonium citrate (green) 0.0021%, EDTA 0.0001%, potassium phosphate dibasic 0.0039%, magnesium sulfate heptahydrate 0.0075%, sodium carbonate monohydrate 0.002%, each concentration in [%w/v]. Optimal density was measured at 750 nm with photometer (spectral photometer Hach Lange Xion 500). At $OD_{750} = 1$ the precultivation sample was diluted in BG11 media to $OD_{750} = 0.01$. Samples were taken each 24 hours. Cell growth was recorded as increase in OD_{750} and the potential ethanol production via cyanobacteria was measured with HPLC analyses. As negative controls the BG11 media and wild type *Synechococcus elongatus* were used.

RESULTS AND DISCUSSION

Three different vectors were constructed via PCR from the template construct pLOI295 or amplified direct from the genomic DNA of *Z. mobilis* and named P6C, P6PA and P6PAA. The vector P6C contains the original cassette encoding *adhB* and *pdc* gene from *Z. mobilis* (see Fig. 1).

This cassette was separated in the vector P6PA using PCR and ligation and each gene had its own promoter and terminator (see Fig. 2). One additional gene, *adhA* from *Z. mobilis*, was introduced using PCR and ligation, the resultant vector was named P6PAA (see Fig. 2).

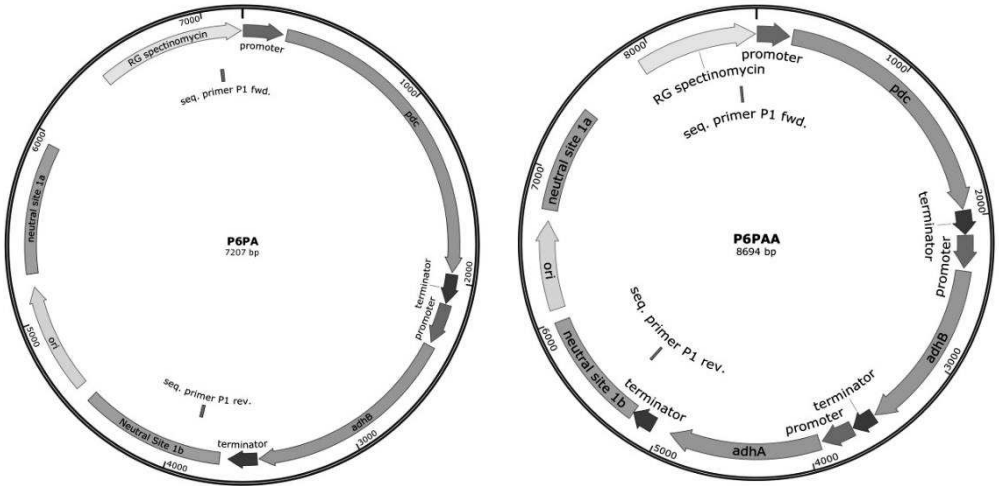

Figure 2. The map of the vectors 6PA and P6PAA – The P6PA contained the gene *adhB* and *pdc* gene consecutively, each with its own promoter and terminator. The vector P6PAA included an additionally gene, *adhA*.

Each vector was controlled via sequencing and transformed into *Synechococcus elongatus* cells. After several days many colonies were observably on the agar plate.

Exchange the Fig. 2 (plus text).

Ten colonies were picked, the successful transformation of the *Synechococcus elongatus* cells was controlled via PCR using the cloning primers and cultivated in 6 mL BG 11 media (in 6 wells plate) to check the growth. Well growing colonies were transferred to 500 mL shake flasks containing 100 mL media. Media and wild type *Synechococcus elongatus* cells were used as negative controls. Each 24 hours samples were taken and the potential bioethanol production were checked via HPLC method. Furthermore the optical dense was measured from each sample. Wild type *Synechococcus elongatus* cells grown faster than the transformed *Synechococcus elongatus* P6C cells (see Fig. 3).

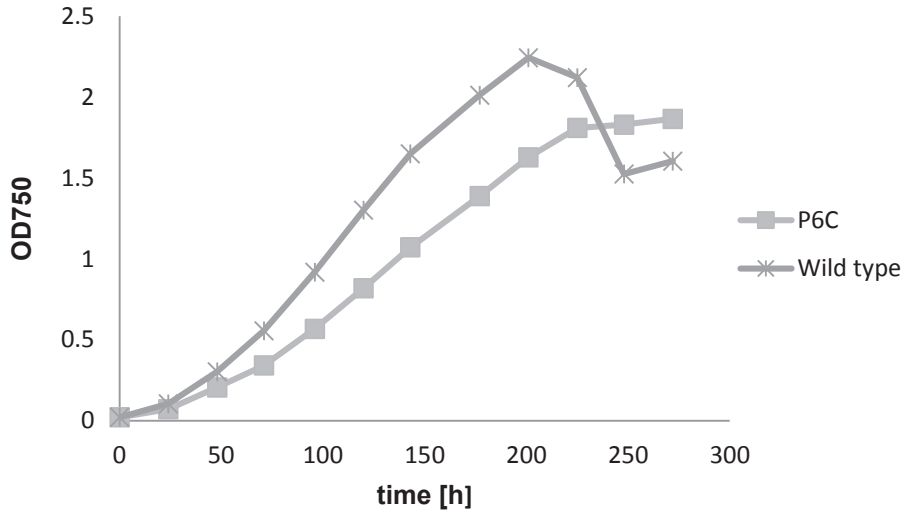

Figure 3. Growth curve of wild type and transformed *Synechococcus elongatus* P6C cells at OD 750 nm.

The sharp decrease within the growth curve after about 220 hours from wild type *Synechococcus elongatus* was generated maybe due to decline of trace elements like iron but must be clarified in detail. Bioethanol was only detected in media from *Synechococcus elongatus* P6C cells See Fig. 4). Also the other transformed *Synechococcus elongatus* shown the same growth curve like *Synechococcus elongatus* P6C cells (data not shown) but produced no bioethanol.

The bioethanol production from *Synechococcus elongatus* P6C cells increased up to a maximum of 0.05 g L^{-1} after about 250 hours (see Fig. 4). In comparison the strain ABICyano1 from the company Algenol reached a the maximum rate of 0.552 g L^{-1} bioethanol after one day (patent 20140178958). Also direct comparison with other data is not possible – our strategy was never use from other researcher.

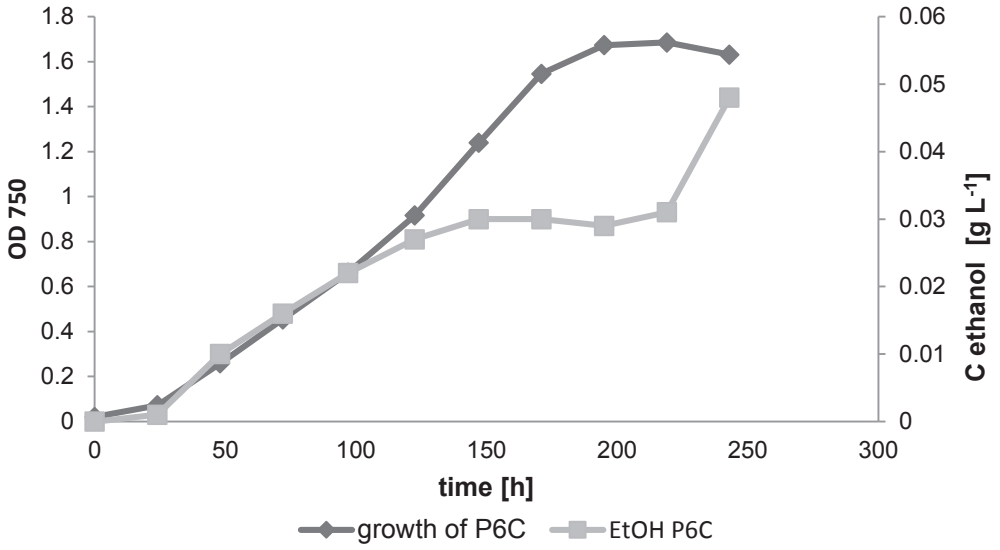

Figure 4. Growth curve of transformed *Synechococcus elongatus* P6C cells at OD 750 nm. Parallel potential bioethanol production of each sample was measured with HPLC.

Anyway here the experiments were done in a very simple way – no fixed closed system (only closed with cotton wool) in a shaker flask – no reactor with regulation of the light, CO_2, pH value and nutrients within the media like the system from the company Algenol. Therefore improvements concerning the *Synechococcus elongatus* cultivation are underway in our lab and further genetic improvements must be performed to increase the bioethanol production.

CONCLUSIONS

Diverse products like bioethanol can be manufactured from cyanobacteria after genetic manipulation, but also glucose/fructose or lactate (for further information see Ducat et al., 2010). But the productivity must be increased for industrial scale up – diverse different strategies were done concerning the genetic manipulation of cyanobacteria – other kind of vectors, other promoter and other strain of cyanobacteria and so on. Here we tested the strong constitutive promoter of psbA1 gene (ending photosystem II protein D1) and diverse combinations of the gene pyruvate decarboxylase (*pdc*), alcohol dehydrogenase B (*adhB*) or *adhA* from *Z. mobilis* or the gene cassette (original from Lonnie O. Ingram). Only the *Synechococcus elongatus* P6C cells transformed with the vector including the gene cassette produced bioethanol at high level after about 250 hours at suboptimal cultivation condition. Furthermore technical and genetic improvements must be done for industrial scale up.

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