Evaluation of the inline stripping of ethanol during cyanobacteria cultivation in a bubble column bioreactor

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Abstract. Cyanobacteria are oxygenic phototrophic microorganisms capable of photosynthesis. In this redox reaction driven by light energy, carbon dioxide and water are converted into chemical energy in form of carbohydrates and oxygen. The output of this process is restricted by product inhibition from the bioethanol. Here, we evaluate a method of ethanol stripping in a bubble column for perspective use for determination of ethanol production rate of engineered cyanobacteria. The knowledge about the amount of condensation and recovery rate combined with HPLC measurement for ethanol determination can be used to specify the real amount of produced ethanol (absolute) by cyanobacteria in the used bioreactor. Stripping and recovery rate are depending on several parameter like flow rate, initial ethanol concentration, condensation temperature etc. Due to the high influence of these parameters they have to be supposed to be static regarding to the degrees of freedom.

To evaluate the system different ethanol concentration were testet for stripping and determination of recovery rate. As the stripping rate was much higher compared to the ethanol production rate with our Synechococcus elongatus PCC 7942 the medium was spiked with ethanol to varying concentrations of 0.5, 1 and 2% v/v. It could be shown that ethanol could be removed quantitavely. Removal rates of 97–98% were reached with initial ethanol concentrations of 5 g L^{-1} to 20 g L^{-1}. The results demonstrated determination of ethanol in the exhaust air stream and quantitavely recollection by cultivating engineered *Synechococcus elongatu*s in bubble column bioreactors.

Key words: Bioethanol, stripping, inline down-streaming, cyanobacteria, bubble column bioreactor.

INTRODUCTION

Renewable energies and especially biofuels play an important role to ensure energy sustainability (Savvanidou et al., 2010). Due to the increasing dependency on crude oil imports, and increasing greenhouse gas emissions, there´s an increased need for alternative ways of energy supply. Biofuels like bioethanol or biodiesel actually represent the most prominent technical option because of the possibility of blending with fossil fuels and using without major adaptations of cars (Gnansounou, 2010). Advanced biofuels, such as those made from straw, waste and algae, provide higher greenhouse gas emission savings with a low risk of causing indirect land-use change, and do not compete directly for agricultural land used for food and feed production. Therefore, the EU

encourages greater research in, and development and production of advanced biofuels as they are currently not commercially available in large quantities.

The Directive (EU) 2015/1513 of the European Parliament relating on the promotion to the use of energy from renewable sources regulates the production of biofuels. The amount of biofuels and bioliquids produced from cereal and other starch-rich crops, sugars etc. grown as main crops primarily for energy purposes on agricultural land is capped to 7% of the total amount of transport fuels.

Today the so called first generation biofuels (fuels derived from sugar cane or starchy crops) are produced worldwide. Bioethanol is produced by fermentation and concentrated by distillation. The world bioethanol production was expected to reach 100 billion liters in 2016 (Kahr et al., 2013). Over the last decade, research on advanced biofuels like second generation bioethanol made great progress. The technology for biofuel production derived from straw is now available and the first production site with a capacity of 50.000 tons per year was opened in Italy in 2013 (Novozymes, 2013).

Microalgae and cyanobacteria offer great potential as platform for production of natural and high value products like pigments, proteins and biofuels. Their fast growth, low production cost regarding simple media composition in combination of using light and CO_2 to generate biomass and value products make them ideal as production systems (de Farias Silva & Bertucco, 2016; Yan et al., 2016). Additional to optimization of cultivation parameters, like light and media for high yield production, genetically engineering of these simple structured microorganisms offer additional product diversity and higher product yields. For bioethanol production with microalgae and cyanobacteria existing biochemical pathways are used for more subjective and efficient production (de Farias Silva & Bertucco, 2016). Here, integration of the genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) from *Zymomonas mobilis* enable the conversion of pyruvate to ethanol (Dexter et al., 2015). For microalgae, viability of industrial process for biodiesel production was shown but a suitable process for bioethanol has still to be found (de Farias Silva & Bertucco, 2016). In cyanobacteria different gene constructs and various promoters were tested for optimized ethanol production. Up to date, Gao et al. (2012) achieved the highest ethanol production yield with 5.5 g L^{-1}.

These prokaryotes are fast growing organisms and utilize solar energy and CO_2. First, these prokaryotes must be genetically manipulated for ethanol production. This was shown by several authors (Dexter et al., 2015; Pfannerer et al., 2016). We have modified the photosynthetic prokaryote *Synechococcus elongatus PCC 7942* using homologous recombination to introduce the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase B (*adhB*) genes of *Zymomonas mobilis* into the organism's genome. These cyanobacteria grow in fresh water, seawater or even in wastewater. Both genes were expressed under the control of the strong constitutive psbA1 promoter (encoding photosystem II protein D1).

The fermentation process of high productive engineered *Synechococcus elongatus PCC 7942* is restricted by the desired end product bioethanol (Nozzi et al., 2013). We present a method for an inline downstreaming process to strip the ethanol produced during fermentation of Synechococcus elongatus PCC 7942. The inhibiting effects of ethanol could be avoided and the product ethanol can be collected by this method at the same time. The inline stripping of ethanol from stirred tank reactors was described by Hwai-Shen (1990), Hsien-Wen (1991) and more recently by Amenaghawon (2010). It

has been shown that inline stripping of ethanol can be a valid way to remove and concentrate the product from fermentation processes.

In conventional bioethanol production, ethanol is generated by yeasts in an anaerobic process without any aeration. Ethanol produced is separated at the end of the process commonly by means of rectification. We describe an inline process which allows the continous separation of the produced ethanol throughout the fermentation process in bubble column reactors. The procces can be summarized as follows: Cyanobacteria must be provided with a CO_2 as carbon source. Routinely, this is provided by flushing the fermentation reactors with compressed air.

Stripping of ethanol in general is not a new invention and was already desribed too for CO_2 stripping from high concentrations (8–9% v/v ethanol in wine) in bubble columns by Silva et al. (2015). The aim of the work was to evaluate the stripping method so far in our fermentation system to define the exact stripping conditions for fermentation of high producing (genetically modified) cyanobacteria.

We have utilized a commercial bubble column reactor for fermentation of bioethanol from *Synechoccus elongatus* and examined the stripping of the ethanol produced by the bacteria. As a result, a stable fermentation process can be designed which prevents the inhibition of the Cyanobacteria by the toxic ethanol.

MATERIALS AND METHODS

Cultivation of *Synechococcus elongatus PCC 7942*

Wildtype *Synechococcus elongatus PCC 7942* was used for fermentation to evaluate inline stripping process with added ethanol in different concentrations in a bubble column bioreactor. Pre culture was incubated in 100 mL Erlenmeyer flasks on 20 m LBG11 medium as described by CCAP (Culture Collection of Algae and Protozoa) for 48 hours. For agitation a Gerhard Laboshake THO 500 was used. Incubation conditions were 34 °C, 150 rpm at PAR of 100 μmol m^{-2} s^{-1}. Bubble column bioreactor was inoculated with 100 mL of preculture.

Fermentation: For fermentation a bubble column bioreactor was used (Fig. 1). The reactor consists of a circular glass tube, working vol. 1,000 mL to 1,300 mL (length 540 mm, diameter 60 mm). Temperature was adjusted to 24 °C, PAR was 100 μmol m^{-2} s^{-1}. The reactor was flushed with compressed, sterile filtered (0.2 μm, PA) air from the bottom. The flow rate V varied from 0.5 L^{-1} to 2.0 L^{-1}. Experimental conditions for the examination of stripping effects are shown in Table 1.

Stripping experiments

Ethanol stripped from the medium through flushing was cooled via a Dimroth condenser, connected to a Thermo Fisher Lauda 600 + 610 thermostat (Fig. 1). Temperature of cooling water was 5 °C. The production rate of ethanol by the modified *Synechococcus elongatus* can`t yet be predicted exactly at the actual state. In addition the ethanol in the system applied is not recovered completely as shown later (Fig. 5). So an exact mass balance is difficult to generate. Therefore a non ethanol producing wild type of *Synechococcus elongatus* was used an the fluid was spiked with ethanol of varying concentrations of 0.5, 1 and 2% v/v. Experimental conditions for the examination of ethanol stripping effects are shown in Table 1.
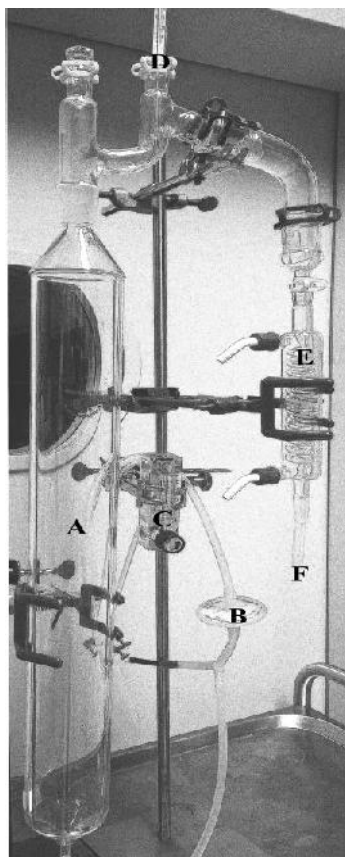
Figure 1. Bioreactor including inline ethanol stripping; A: bubble column reactor; B sterile filter disc for aeration; C: flow meter; D: thermometer; E: Dimroth condenser connected to Thermo Fisher Lauda FB 600+610; F condensate connected to volumetric device; (image post processed for better visualization).

Table 1. Experimental conditions for examination of ethanol stripping effects

Series	cEtOH at Start	Air Flow rate
1	2% v/v	2 L^{-1}
2	1% v/v	2 L^{-1}
3	0.5% v/v	2 L^{-1}
4	1% v/v	1 L^{-1}
5	1% v/v	0.5 L^{-1}

Ethanol Determination per HPLC

Ethanol concentration in the stripped condensate was determined by HPLC, using an Agilent Technologies 1200 Series connected via Agilent 35900 AD converter to a refractive index detector from Jasco (RI 2031plus). As chromatographic column a Varian Metacarb 87 H (length 300 mm, diameter 7.8 mm) at 65 °C was applied. Sulfuric acid (c = 5 mmol L^{-1}) as eluent with an isocratic flow rate of 0.8 mL min^{-1} was used. The injection volume was set to 80 µL. For calibration, the method of external standard was used in a range from 10 to 200 mg L^{-1} ethanol (VWR, p.a., < 99,9%). For low target concentrations between 5 and 10 mg L^{-1} of ethanol, the quantification was performed with the standard addition method. The data acquisition was performed per refractive index detection with the software Agilent Chemstation V 03.04 b.

RESULTS AND DISCUSSION

To evaluate the inline stripping process of ethanol in a bubble column bioreactor a cultivation of cyanobacteria Synechococcus elongatus PCC 7942 wildtype was performed. Different air flow rates and initial ethanol concentrations were used, the recovery rate of ethanol was determined.

Table 1 shows the experimental conditions for the different sets of experiments. Ethanol was added to the medium up to 2% (w/v) (Table 1), different air flow rates (between 0.5 and 2 L min^{-1}) were tested. These concentrations are much higher than actually reached with Synechococcus elongatus PCC 7942 (Pfannerer et al., 2016) but are regarded as a target which should be achievable with further genetic optimization of the strain. The process was monitored for 8 days:

In the first series of experiments a fixed air flow rate of 2 L⁻¹ and ethanol concentrations of 0.5, 1 and 2% (v/v) were examined. The removal of ethanol increased exponentially by increased initial ethanol concentrations (Fig. 2). After 96 h of incubation ethanol was below 0.1% v/v ethanol for all three initial concentrations. The specific removal is not directly depending on the actual concentration of ethanol in the bioreactor. Therefor the method is working for any initial ethanol concentration.
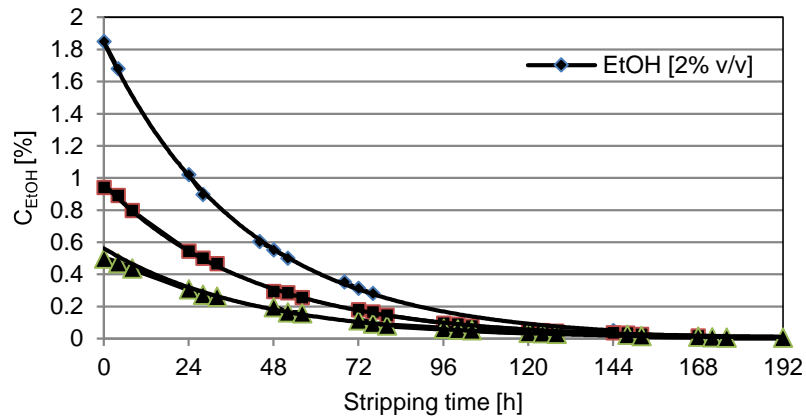

Figure 2. Ethanol removal from bioreactor in dependency on initial ethanol concentration. Conditions: air flow rate 2 L min⁻¹, Temp. bioreactor 24 °C, initial ethanol concentration 0.5%, 1%, 2%.

The specific ethanol removal rate (ethanol removed in $g\ L^{-1}\ d^{-1}$) from the reactor is shown in Fig. 3. The daily removal rate is about $10\ g\ L^{-1}\ d^{-1}$ at ethanol concentrations of approximately $10\ g\ L^{-1}$. This corresponds to $0.4\ g\ L^{-1}$ per hour. The important consequence of this finding is the fact that the ethanol production rate of the cyanobacteria may be $0.4\ g\ L^{-1}$ per hour or lower if the ethanol concentration may not exceed this (possibly toxic) concentration (Dexter & Fu, 2009).
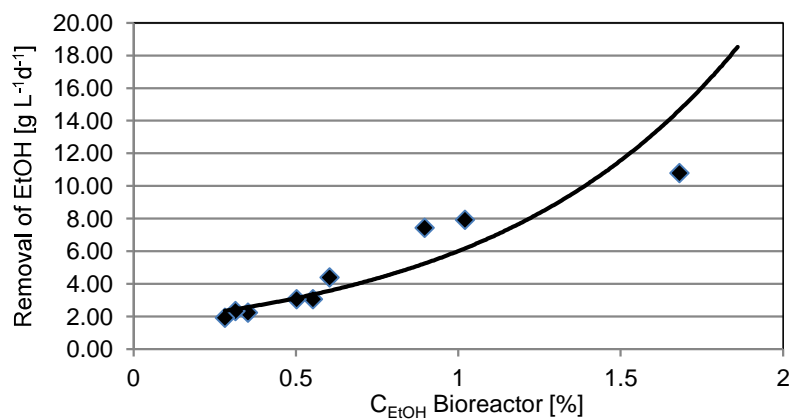

Figure 3. Specific ethanol removal rate dependent on actual ethanol concentration in the bioreactor. Conditions, airflow rate 2 L min⁻¹, temp. Bioreactor 24 °C, initial ethanol concentration 2%.

Fig. 4 shows the influence of different air flow conditions on the stripping of the ethanol. In all experiments the starting ethanol concentration was 1% ethanol (v/v). At an airflow rate of 2.0 L min^{-1} (which is definitely more than needed for the supply of Synechococcus elongatus PCC 7942 with carbons source and also higher than needed for turbulence in the bioreactor) the ethanol concentration fell below 0.3% (v/v) after 48 h of incubation and fell under 0.02% after 144 h. The ethanol removal at an air flow rate of 0.5 L min^{-1} is definitely too low to ensure not toxic effects of ethanol produced by a high productive strain of Synechococcus elongatus PCC 7942. An ideal air flow rate have to be fitted to the ethanol productivity of the engineered strain and its sensitivity against the end product ethanol. Fig 4. shows respectively, ethanol production could be determined in any experiments using bubble column bioreactors.
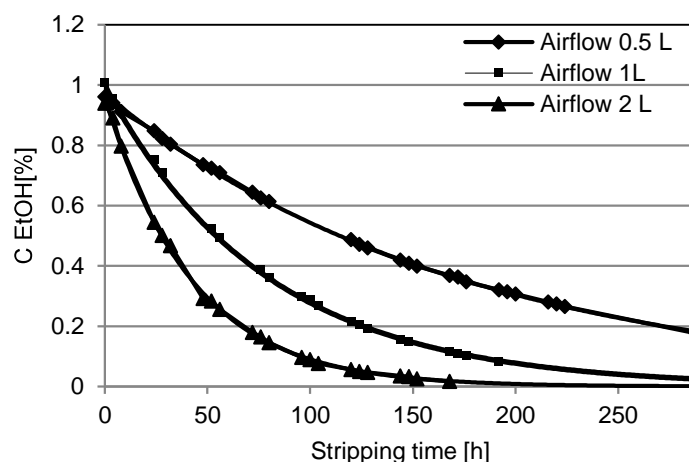

Figure 4. Ethanol concentration versus stripping time from bioreactor. Conditions, airflow rate 0.5, 1, 2 L min^{-1}, Temp. bioreactor 24 °C, initial ethanol concentration 1%.

CONCLUSIONS

Stripping of ethanol constantly throughout the fermentation process using bubble column bioreactors is a suitable and certainly cost effective technique for the separation of ethanol produced by cyanobacteria. More than 97% can be removed ad moderate volumetric air flow rates which are anyway needed for a bubble column bioreactor. So the end product inhibition of a high productive engineered strain by the ethanol is avoided. A challenge which has to be solved is the modest recovery rate of the ethanol. The stripped ethanol was collected via low temperature condensation. Experiments were performed with variation of flow rate and ethanol concentration. Elimination rates of 97–98% were reached with initial ethanol concentrations of 20 g L^{-1} respectively 10 g L^{-1}. The daily removal rate of about 10 g L^{-1} d^{-1} at ethanol concentrations 1% and of 20 g L^{-1} d at ethanol concentrations 2% ethanol (v/v) should be high enough for state of the art ethanol concentrations (Woods et al., 2012). The important consequence of this finding is the fact that the ethanol production rate of the cyanobacteria can reach 0.4–0.8 g L^{-1} per hour without reaching an inhibiting ethanol concentration. A major

finding is that aerated column reactors are not all suitable for kinetic studies of biological ethanol production.

The experiments show a further and very important consequence. If laboratory and pilot scale fermentations for the production of ethanol in bubble column reactors are carried out, the measurement of the ethanol concentration within the medium is more or less useless, because the ethanol produced is stripped out. A possibly low production will be faked. To circumvent the problem the measurement of the ethanol stripped in the air flow (for example by means of IR detection) is obligatory. In experiments with agitated flasks the amount of ethanol produced must be examined in the head space.

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