INTERREG CZECH REPUBLIC - AUSTRIA

ATCZ15 - Společné česko-rakouské centrum řasových biotechnologií

SUMMARY RESEARCH REPORT

Period	2
Activity	Implementation T1.2
	Determination of cultivation condition
Name	**Characterisation of strains - larger scale**

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Strains:

- *Synechocystis sp.* PCC6803-NIX

Aim:

Evaluation of the tolerance of *Synechocystis* to ethanol.

Experiments:

- Growth rate (cell number)
- Biomass production (dry weight)
- Ethanol tolerance

Culturing conditions/regimes:

- Cultivation volumes: 300 ml in 400 ml cylinders
- Initial concentration: ~0.3 gL^{-1} *Synechocystis*
- Light intensity: ~70 µmol photons m^{-2} s^{-1} inside exposed wall surface of temperatedwater bath (glass reservoir) using cosine-corrected light sensor
- Continuous light
- Temperature: 26-28 °C
- Air bubbling volume (L min^{-1}): 0.25 L/min
- CO_2 % supply:1.5 % CO_2in air (v/v)
- Culture media:BG 11
- Ethanol concentrations: 0.1 M, 0.2 M, 0.4 M, 0.8 M

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Shrnutí v češtině

Vzhledem k záměru projektu bylo nutné ověřit toleranci kultury *Synechocystis* PCC6803 k produkovanému etanolu, který může způsobovat inhibici růstu. Na základě informací v literatuře byly zvoleny koncentrace etanolu od 0.5 do 4 % (v/v).

Bylo zjištěno, že nízká koncentrace etanolu (0.5 %) stimuluje růst kultury divokého kmene a toleruje koncentraci do 1 % (v/v) etanolu, ale dochází k omezení počtu buněk; nicméně při koncentraci 2 % již dochází k omezení růstu. V kontrolní sérii válečků bylo porovnáváno hromadění etanolu v mediu bez kultury, aby bylo možné odhadnout, jak je etanol metabolizován sinicemi, případně jaké jsou ztráty odpařením ze suspenze. Pro tyto pokusy byly testovány metody stanovení fotosyntetické aktivity (pomocí fluorescence chlorofylu) a vypracována metoda detekce etanolu v suspenzi pomocí kapalinové chromatografie.

Summary

Due to the project objectives, it was necessary to verify the tolerance of the Synechocystis PCC6803 culture to the ethanol potentially produced by mutants to the culture which can inhibit growth. Based on information in the literature, ethanol concentrations of 0.5 to 4% (v / v) were tested. Low ethanol concentrations (0.5%) were found to stimulate the growth of wild-type Synechocystis culture. The cultures tolerated up to 1% (v / v) ethanol, but the number of cells was reduced; however, at a concentration of 2%, growth was already inhibited. In the blank series of cultures, the accumulation of ethanol in culture-free media was determined to estimate how is ethanol metabolized by cyanobacteria, or what is the lost by evaporation from the culture suspension. In these experiments, the methods for the determination of photosynthetic activity (by chlorophyll fluorescence) were tested and procedure of ethanol detection in suspension by liquid chromatography was developed.

Experimental scheme

- Cultivation period:Day 1:17.11.2017., Day 5:21.11.2017
- 300 ml culture in 400 ml glass cylinders
- Duplicates (2 parallels) + 1 blank/control (without culture) for each ethanol concentration
- Initial ethanol yield was added every day (1-5 days):

Day 1

- take samples for OD (1 ml), DW (3x2 mL), CN (0.5 mL)
- measure control - Fv/Fm (DCMU), RLC, OJIP (5 mL), EtOH assay (10 mL), carbohydrate (2 mL)

Start of light - 8.00 h

-1 h of acclimation to light and T

- 9.00 h - add EtOH according to table below

14.00 h – measure all cylinders with culture - Fv/Fm (DCMU), RLC, OJIP (5 mL),

- take samples for EtOH (10 mL) and carbohydrate (2 mL)

Day 2

- 8.00h add water to fill up evaporation
- take samples for OD (1 ml), DW (3x2 mL), CN (0.5 mL)
- measure control - Fv/Fm (DCMU), RLC, OJIP (5 mL),
- take samples for EtOH assay (10 mL), and carbohydrate (2 mL)
- 10.00 h - add EtOH according to table below

Addition of 100% EtOH to 300 mL cultivation cylinder

mL of 100% (v/v) EtOH	% (v/v) in culture	M
0	0	0
1.5	0.5	0.098
3	1	0.18
6	2	0.35
12	4	0.70

Density of 100% EtOH – 0.79 kg/L
Mr EtOH =36

Day 3-5
Repeat the same procedure as on Day 2
Day 5 afternoon – end of experiment

Experimental set-up:

Methods

Dry weight measurement:

Dry weight was measured every day. 2 ml samples were taken from each cylinders and stored in Eppendorf tubes at -20 °C every day until utilization. Wet biomass was obtained by centrifugation in pre-weighted Eppendorf tubes. The cell pellets were dried at 105 °C overnight. The mean of dry weight was calculated from triplicates.

Cell counting

Flow cytometer was used to determine cell number

Assay of Ethanol concentration

800 μl samples were taken from *Synechocystis* cultures and blank/control (without *Synechocystis*) cultures every day. The samples were always taken before another addition of ethanol. They were centrifuged and the supernatants were stored at -20 °C until measuring. Ethanol content was measured by LC. 20 μl was injected on a Hamilton PRP-X100 column when5mM $H_2SO_4$was used as mobile phase and the flow rate was set to 1.5 mL min^{-1}. Ethanol concentrations were calculated from calibration curve.

Results and discussion

Dry weight determination

DW (g / l)	D1	D2	D3	D4	D5
0A	0.522	0.938	0.973	1.280	1.490
0B	0.518	0.833	0.930	1.218	1.470
0.1A	0.533	0.940	0.993	1.440	1.733
0.1B	0.605	0.875	0.908	1.415	1.502
0.2A	0.615	0.847	0.850	1.262	1.418
0.2B	0.668	0.763	0.777	1.235	1.603
0.4A	0.643	0.742	0.665	0.822	1.257
0.4B	0.608	0.652	0.557	0.898	0.662
0.8A	0.660	0.392	0.173	0.410	0.308
0.8B	0.637	0.505	0.280	0.362	0.312

Table 1: dry weight changing in *Synechocystis* cultures with addition of ethanol.

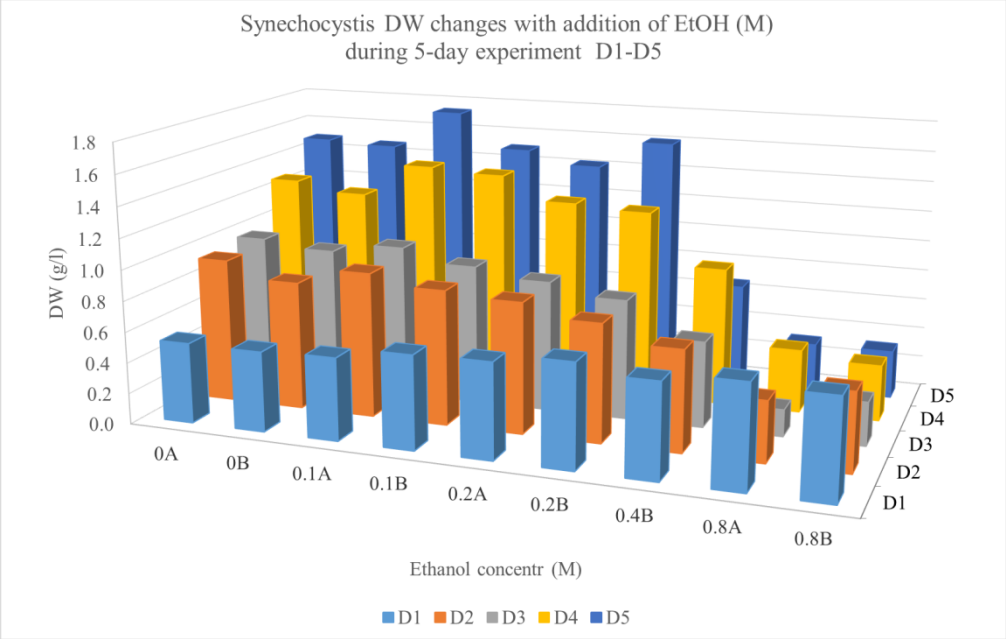

Figure 1: Changes of dry weight in *Synechocystis* culturesas a function of ethanol concentration

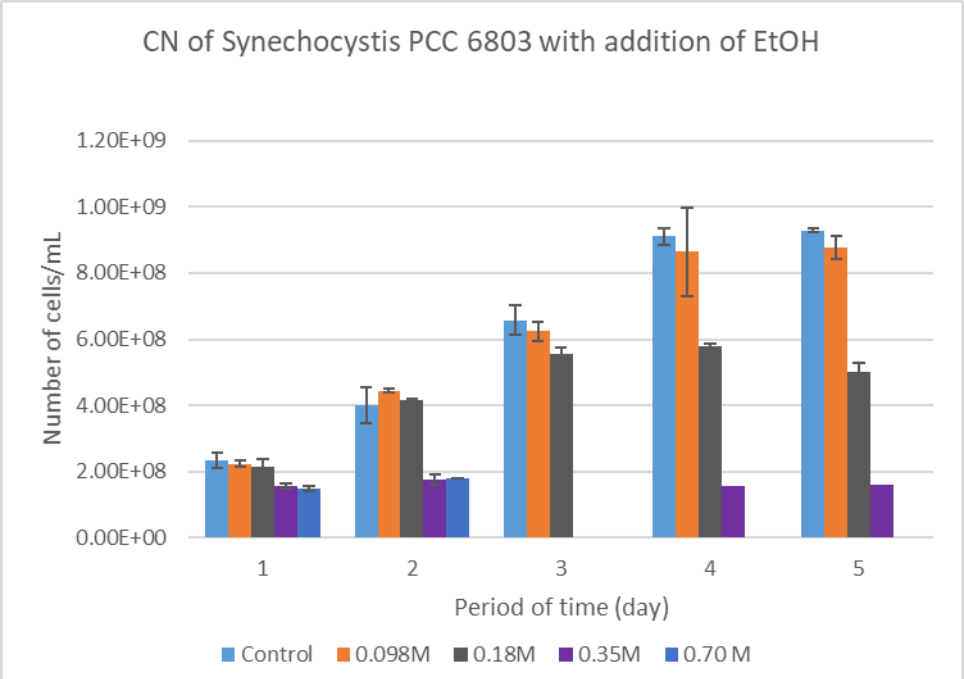

Figure 2: Cell numbers of *Synechocystis* cultures with addition of EtOH

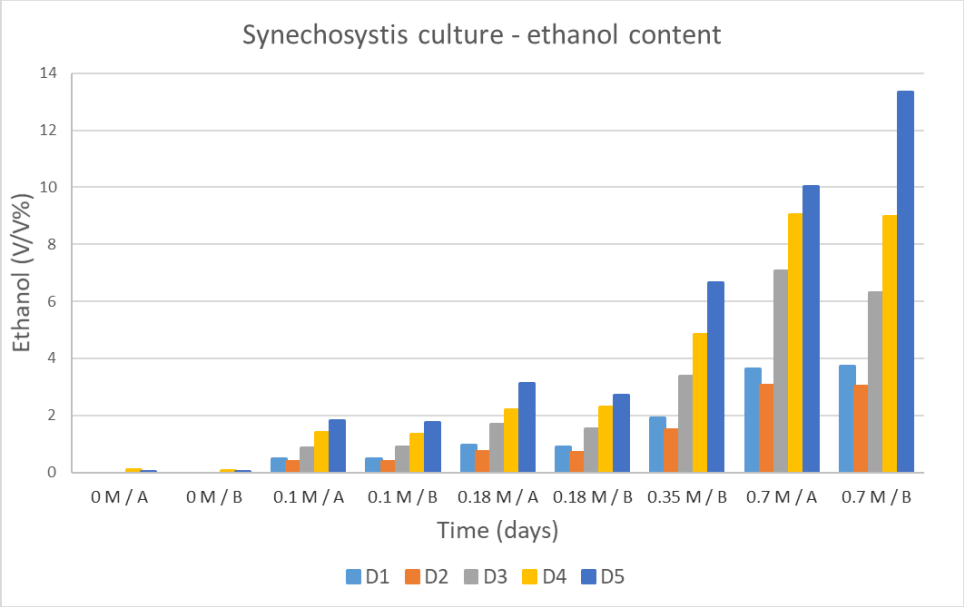

Figure 3: Ethanol content measurements in *Synechocystis* cultures

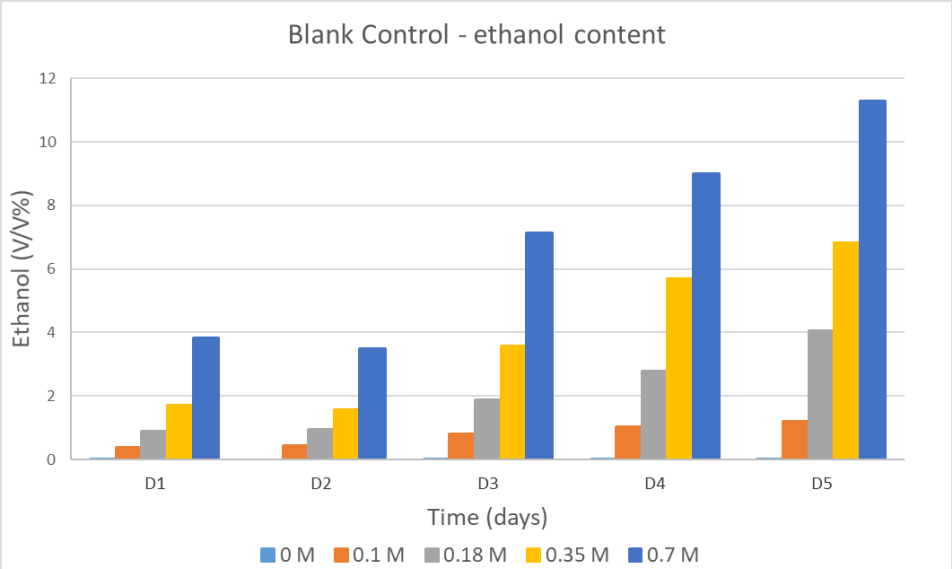

Figure 4: Ethanol yields in blank/control samples (only BG11 media + ethanol)

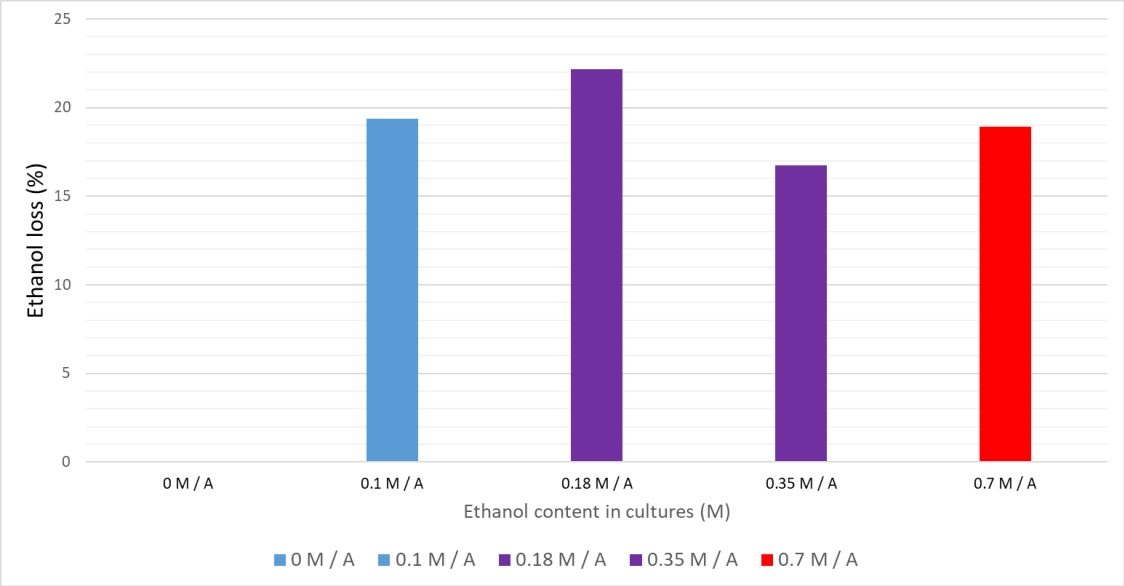

Figure 5: Estimated ethanol loss from the cultures

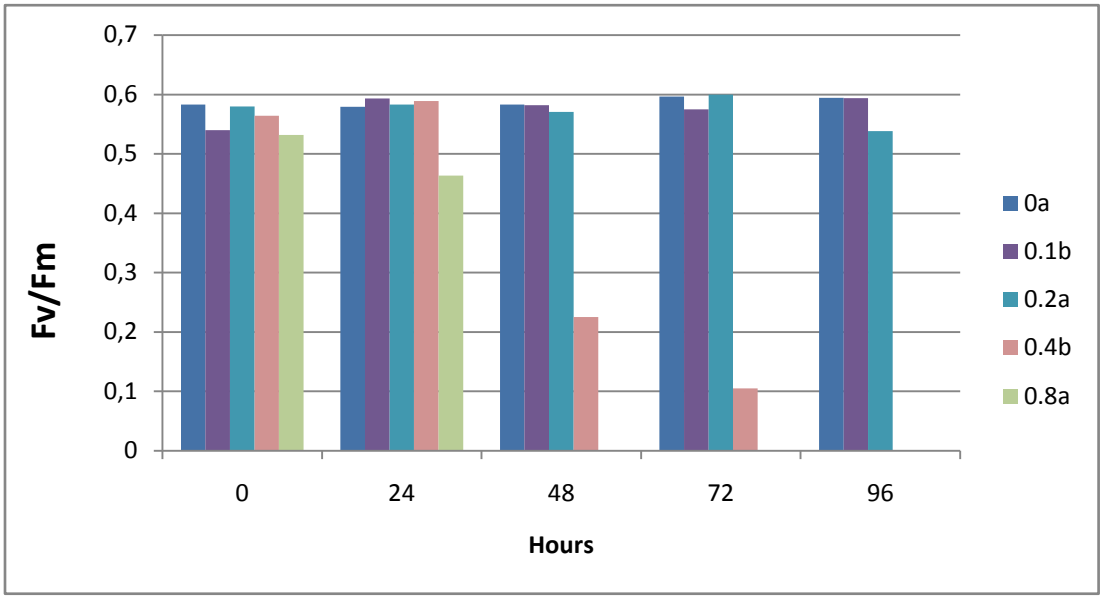

Fig. 6 Changes of maximum photochemical yield in Synechococcus cultures exposed to various concentrations of Ethanolduring 5-day experiment

Dry weight

Biomass production was followed by dry weight measurements (Fig. 1). Low concentration of ethanol (0.1 M) stimulated the growth of Synechocystis culture as compared to the control. In the rest of the cultures, in which the initial ethanol concentrations were 0 M, 0.1 M and 0.18 M the biomass growth was continuous during the five days, although some slow-down was observed in the cultures containing 0.18 M ethanol. In the cultures in which the initial ethanol concentration was 0.35 M the dry biomass increased until the third day when the culture was not able to cope/metabolise with the ethanol concentration. After that biomass yield grew, but on the last day in one of the culture biomass loss occurred.In the cultures in which the initial ethanol concentration was 0.8 M the growth was completely stopped.

Cell number

The cell counting showed that the 0.18 M ethanol content already caused some changes as it reduced cell number by about 30% on Day 4 of culturing although growth was not significantly influenced (Fig. 2). We suppose that cell division was inhibited in the presence of ethanol.In the culture containing 0.35 and 0.7 M ethanol cell numbers wereseverely decreased.

Ethanol concentration

Ethanol content was determined by LC (Fig. 3). The loss of ethanol was observed in previous studies and also during the current experiment (Fig. 5). The ethanol loss in 24 hours is showed between the first and the second day was calculated from the content in the culture and the blank (Fig. 3 vs. Fig. 4). To balance the ethanol loss, the initial ethanol amountswere added every day which caused ethanol yield raising in the cultures. Besides, strong ethanol loss was observed in the cases of 0.4M/A and 0.8M/A. We assume that it was caused by bacterial contamination.

Photosynthetic activity changes

The control culture grew well and showed good activity of photosynthesis measured by chlorophyll fluorescence techniques (maximum photochemical yield Fv/Fm = 0.55-0.60, electron transfer rate 20-30 µmol of m^{-2} s^{-1} electrons). Fast fluorescence induction curves showed a typical coursethat was changed when *Synechocystis* culture was subjected to, for example, high irradiation stress. In this case, the fluorescence variables measured at 2-3 msec and 30-50 msec on the induction curve significantly increased.

Conclusions

1. Synechocystis cells can divide until the ethanol concentration reaches 1.5 %.
2. Synechocystis cells can grow approx. 1.5 % ethanol concentration, maximum ethanol concentration was 2.5%
3. Evaporation causes ethanol loss
4. Cultures are sensitive for bacterial contamination
5. Bacterial contamination can cause strong ethanol loss