Butanol Biosynthesis From *Bacillus subtilis*

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Introduction

Bio-Butanol is a second generation alcoholic fuel with a higher energy density and lower volatility than ethanol that is currently produced using anaerobic culture of *Clostridia spp*.. While efforts are focused on improvement of the yield from butanol fermentation, the productivity is still hindered by the effects of butanol cytotoxicity.

Butanol is known to cause an increase in membrane fluidity by intercalating in the phospholipid membrane and breaking hydrogen bonds between lipid tails, resulting in a loss of membrane potential and decline in cell growth (1).

Aims

1. To construct a *B. subtilis* strain where the butanol biosynthetic genes from *Clostridia* have been introduced.
2. Design a suitable butanol assay and measure the butanol productivity of constructed strains under defined growth conditions.
3. Analyse butanol toxicity and determine whether the inserted constructs had any effect on butanol resistance.

Experiment 1: Strain construction

The B subtilis 168 strain is reconstructed with expression vectors that contain selective antibiotic resistance markers; pdBCS (Spectinomycin), pjT (Erythromycin) and ppA2 (kanamycin). Enzyme encoding genes are shown in Fig. 1.

Positive transformants were isolated on nutrient agar plates containing their antibiotic markers. Single constructs were then transformed again to produce double constructs, and eventually the double constructs were transformed to produce the triple construct BK 1.0 (containing pdBCS, pJT and ppA2).

References

1. Kanno, M., Katayama, T., and Tamaki, H. (2013) Isolation of Butanol- and Isobutanol-Tolerant Bacteria and Physiological Characterization of Their Butanol Tolerance. *Applied and Environmental Microbiology* 79, 6998-7005
2. R.Nielsen, D., Leonard, E., and Yoon, S.-H. (2009) Engineering alternative butanol production platforms in heterologous bacteria. *Metabolic Engineering* 11, 262-273

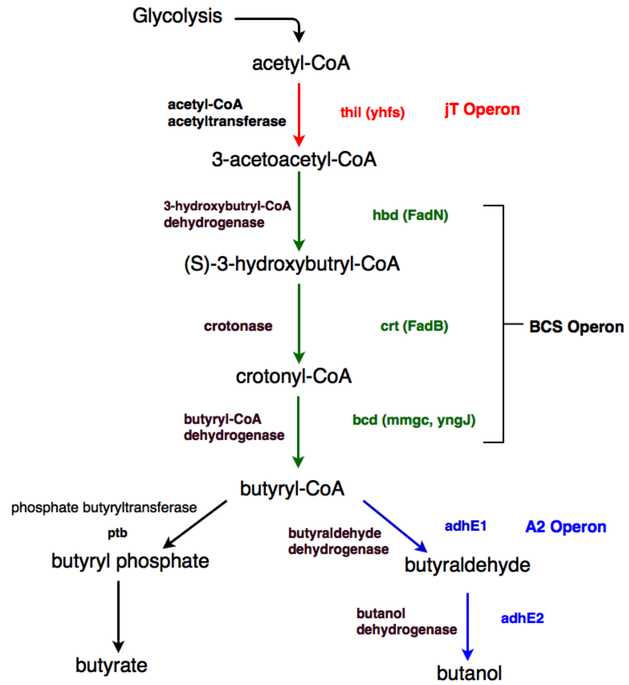

Fig. 1. The acetone-butanol –ethanol (ABE) fermentation pathway of *C. acetobutylicum*. Enzymatic steps to reconstruct the biobutanol pathway are shown. Relevant *C. acetobutylicum* genes are also indicated, while those genes encoding enzymes of homologous function from *B.subtilis* are shown in parentheses.

Experiment 2: Design suitable butanol assay.

Our butanol assay is based on the conversion from NAD+ to NADH. The oxidation of the alcohol substrate (1-butanol) to butyraldehyde will produces a hydrogen ion which will simultaneously transfer to the NAD+ in its specific hydride accepting regions, producing NADH. NADH can be measured by spectrophotometry at OD_{340nm}.

We added a fixed amount of NAD and a fixed amount of alcohol dehydrogenase and a known concentration of butanol (2%, 0.4%, 0.08%, and 0.016%). A formula was calculated from the standard curve. To test the reconstructed strains we grew the strains over-night in minimal media with a fixed amount of food.

Using our butanol assay method, we measured an OD of 0.16 which would equate to 0.028% butanol using the formula, this is 10 times higher than the concentrations obtained in the Nielsen study (2).

Experiment 3: Determine whether the inserted constructs improved butanol resistance.

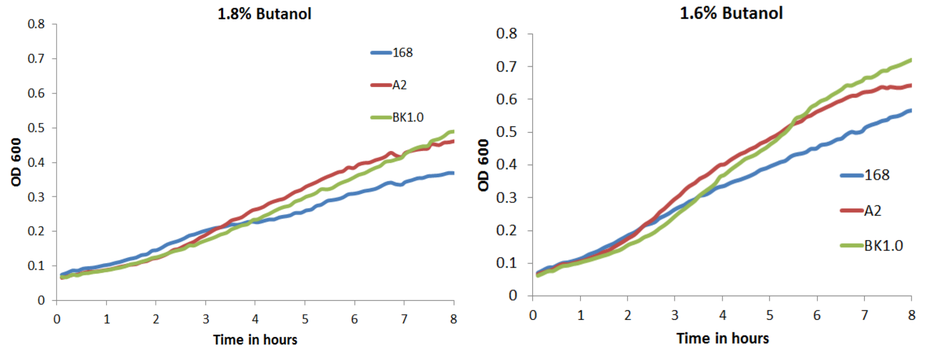

Fig. 2. Comparing the growth curves of *B.subtilis* 168, A2 and BK 1.0 in LB at different butanol concentrations (1.8%, 1.6%). All cultures were supplemented with 1mM IPTG.

In the following experiment, we removed the gene for the part of the metabolic pathway converting butyryl-CoA to butyryl phosphate. *ptb*$^-$ mutants were constructed via gene knockout.

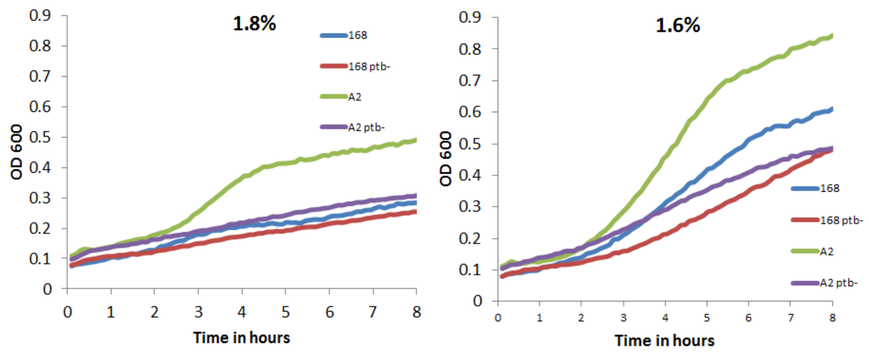

Fig. 3. Comparing the growth curves of *B.subtilis* 168, 168ptb$^-$, A2 and A2 ptb$^-$ in LB at different butanol concentrations (1.8%, 1.6%). All cultures were supplemented with 1mM IPTG.

Conclusion

The results show the reconstructed strains were able to produce 0.028% under the conditions we tested, but the experiment should be repeated using HPLC or gas chromatography to confirm our findings.

Strains containing the A2 construct were more resistant to toxic concentrations of butanol and further experimentation showed that the butanol to butyrate pathway may play a significant part in improving resistance.
Further directions will include employing mutagenic techniques or overexpression of proteins to enhance the butanol resistant phenotype.