The inability of Clostridium beijerinckii to efficiently utilize glycerol, currently experiencing a market glut due to increased biodiesel production is a major impediment to adopting glycerol metabolism as a strategy for increasing NAD(P)H regeneration to mitigate lignocellulose-derived inhibitor (e.g. furfural) toxicity, and improve butanol titer during fermentation of lignocellulosic biomass hydrolysates (LBH). Therefore, metabolic engineering was pursued to enhance glycerol utilization in C. beijerinckii to improve NAD(P)H regeneration and butanol production in furfural-replete LBH. Towards this goal, glycerol catalytic arsenal from the hyper-glycerol utilizing bacterium, Clostridium pasteurianum was cloned and overexpressed in C. beijerinckii. Glycerol dehydrogenase (gldh), the first enzyme in the glycerol catalytic pathway, catalyzes an NAD(P)H yielding reaction, dehydrogenation of glycerol to dihydroxyacetone (DHA) while the DHA kinase-catalyzed reaction yields a glycolytic intermediate (DHA phosphate). As a preliminary step, C. pasteurianum gldh genes – dhaD1 and gldA1 were overexpressed as a fusion construct in an E. coli-Clostridium shuttle vector - pUR460 under the control of constitutive thiolase promoter. The generated strain, C. beijerinckii-gldh was used to conduct batch acetone-butanol-ethanol (ABE) fermentation in a glucose-based medium supplemented with glycerol and 2, 3, 4, 5, or 6 g/l furfural. Fermentation profiles for all furfural concentrations show that C. beijerinckii-gldh accumulated significantly higher cell biomass (30 to 55%) when compared to the empty plasmid control. At high furfural concentrations (5 and 6 g/l), butanol production by C. beijerinckii gldh were 10% and 46% higher, respectively, than the plasmid control ABE concentration and productivity increased by 40.2% and 39.1% with 6 g/l furfural, and glycerol utilization increased by 44% to 70% for all furfural concentrations. Taken together, gldh overexpression in C. beijerinckii improved furfural tolerance and glycerol utilization in C. beijerinckii, thus, we infer that improved NAD(P)H regeneration stemming from glycerol catabolism supplies additional reducing power for efficient detoxification of furfural, which consequently promotes cell growth and butanol production.

**Overview**

- Overexpression of glycerol dehydrogenase, which catalyzes an NAD(P)H yielding reaction of glycerol catabolism, yields additional reducing equivalents for furfural detoxification and butanol production in LBHs.

**Materials and methods**

- We have shown previously in our Laboratory that glycerol supplementation of the growth medium increases C. pasteurianum (glycerol dehydrogenase) wild-type butanol productivity by 10% and 5 g/L furfural challenge and by 46% at 6 g/l furfural challenge. Similarly, ABE production increased to 40% at 6 g/L furfural. The role of furfural detoxification by C. beijerinckii gldh after 2 h of challenge was significantly higher (p<0.05) than the control (not shown).

**Results**

- We identified two glycerol dehydrogenases genes as a fusion protein increased butanol production by 10% at 4 and 5 g/L furfural challenge and by 46% at 6 g/L furfural challenge. Similarly, ABE production increased to 40% at 6 g/L furfural. The role of furfural detoxification by C. beijerinckii gldh after 2 h of challenge was significantly higher (p<0.05) than the control (not shown).

**Fig 5:** DNA gel image showing PCR amplification of C. pasteurianum genes dhaD1, gldA1, dhaK, and generation of fusion constructs (A). Cell growth during fermentation of glucose-glycerol medium by furfural-challenged C. beijerinckii gldh (B-E). At 3, 4, 5, and 6 g/L furfural challenges, cell growth increased by 51.5%, 55%, 46% and 30%, respectively, relative to the control.

**Fig 6:** Butanol and ABE profiles during fermentation of glucose-glycerol medium by furfural-challenged C. beijerinckii gldh. Overexpression of two glycerol dehydrogenases genes as a fusion protein increased butanol production by 10% at 4 and 5 g/L furfural challenge and by 46% at 6 g/L furfural challenge. Similarly, ABE production increased to 40% at 6 g/L furfural. The role of furfural detoxification by C. beijerinckii gldh after 2 h of challenge was significantly higher (p<0.05) than the control (not shown).

**Conclusions and Discussion**

- We have shown previously in our Laboratory that glycerol supplementation of the growth medium increases NAD(P)H regeneration, improves in situ detoxification of furfural and butanol production in furfural-challenged cultures of C. beijerinckii (Ujor et al., 2014).

- The major drawback of the above-stated study was inefficient glycerol utilization by C. beijerinckii. Hence, we sought to address this drawback in the present study.

- By overexpressing two glycerol dehydrogenase genes from a hyper-glycerol utilizing bacterium, Clostridium pasteurianum as a fusion protein in C. beijerinckii, utilization of glycerol improved in the recombinant strain.

**Acknowledgements**

- We acknowledge the financial support from Ohio State University & OARDC, OSU-Agricultural Research and Development Center (Grant Numbers: 2014-51100-22453 and 2014-51101-22456), and Project No. OHIO10320 and Office of Research Technology Transfer. We also gratefully acknowledge the assistance of the Ohio Plant Biotechnology Consortium (OPBC) and the faculty and staff of the Department of Animal Sciences, Ohio State University for their support and encouragement.

**References**