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Metabolic engineering for enhanced furfural tolerance during cellulosic butanol fermentation by glycerol-supplemented *Clostridium beijerinckii*

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Abstract

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 catabolic 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 catabolic 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. pasterianum* gldh genes – dhaD1 and gldA1 were overexpressed as a fusion construct in an *E. coli-Clostridium* shuttle vector - pWUR460 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

- Bioconversion of lignocellulosic-derived sugars to biofuels is hampered by **microbial inhibitory compounds** that are co-generated with sugars during biomass pretreatment (**Fig. 1**). Improving microbial tolerance to LDMICs is crucial to industrial-scale production of cellulosic biofuels and fine chemicals.
- Catabolism of glycerol, a byproduct of biodiesel production, generates NADH, which is critical for detoxification of lignocellulose-derived microbial inhibitory compounds **(LDMICs)** and butanol production by solventogenic clostridia (**Fig. 2**).
- Overexpression of glycerol dehydrogenase, which catalyzes an NADH(P)H yielding reaction of glycerol catabolism, yields additional reducing equivalents for furfural detoxification and butanol production in LBHs.

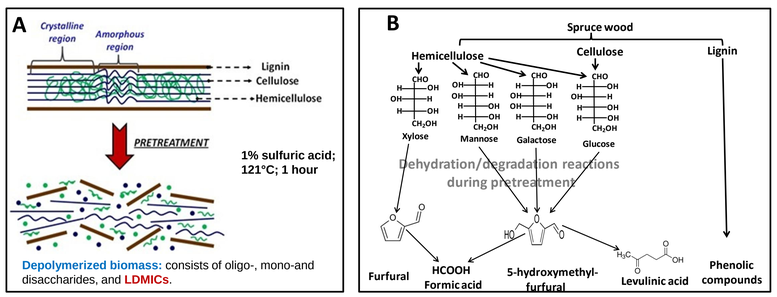


Fig 1: Pretreatment of lignocellulosic biomass. **A**- Conditions and products; **B** – Generation of furanic and phenolic **MICs** by the dehydration and degradation reactions of sugars and lignin during pretreatment.

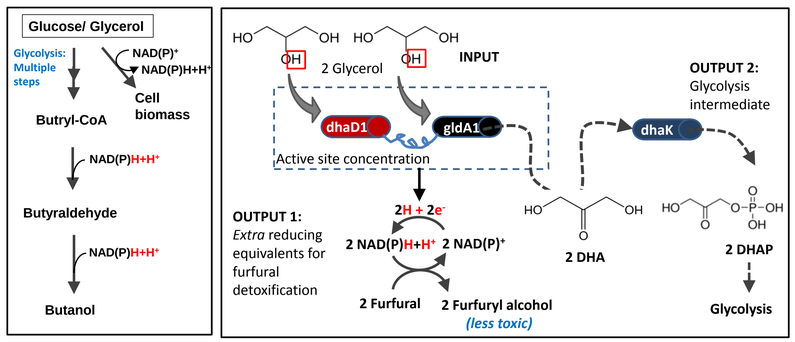


Fig 2: Butanol production and furfural detoxification rely on NAD(P)H. Overexpression of a hyper-glycerol catabolic arsenal from *C. pastuerianum (*glycerol dehydrogenases) generates excess reducing equivalents

Specific aim

- To demonstrate that overexpression of glycerol catabolic arsenal of *C. pasteurianum* (glycerol dehydrogenase) will enhance glycerol utilization and furfural tolerance during the fermentation of glycerol-supplemented and furfural-challenged glucose-based culture of *C. beijerinckii*

Materials and methods

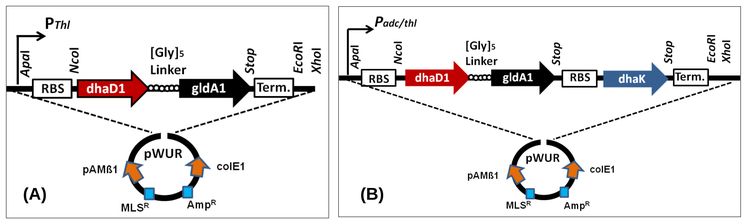
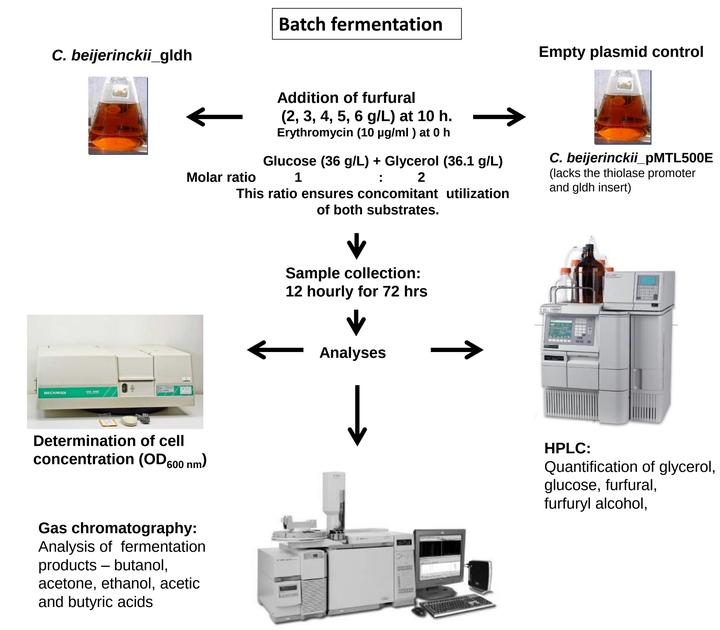


Fig. 3: Construction of recombinant plasmids for systematic over-expression of *C. pasteurianum* DSMZ 525 glycerol catabolic arsenal in *C. beijerinckii* NCIMB 8052. (A) dhaD1+gldA1, (B) dhaD1+gldA1+dhaK. In a preliminary step, *C. beijerinckii* was transformed with pWUR460_dhaD1+gldA1 to yield *C. beijerinckii*_gldh



Results

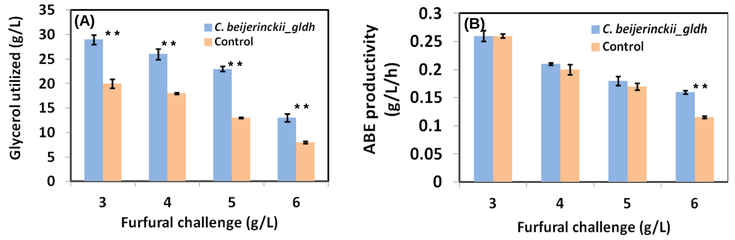


Fig 4: Glycerol utilization and ABE productivity during batch fermentation of furfural-challenged glucose+glycerol medium by *C. beijerinckii*_gldh . At 3 and 4 g/L furfural challenge, glycerol utilization increased from ~20 g/L for the control to ~30 g/L for *C. beijerinckii*_gldh, representing 45% increase. Similarly, at 5 and 6 g/L furfural challenge glycerol consumption increased by 63 and 77%, respectively. Also, ABE productivity increased by 40% at 6 g/L furfural challenge. ** p<0.05

Results (cont'd)

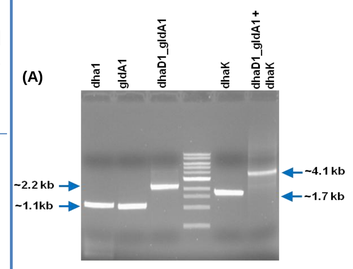
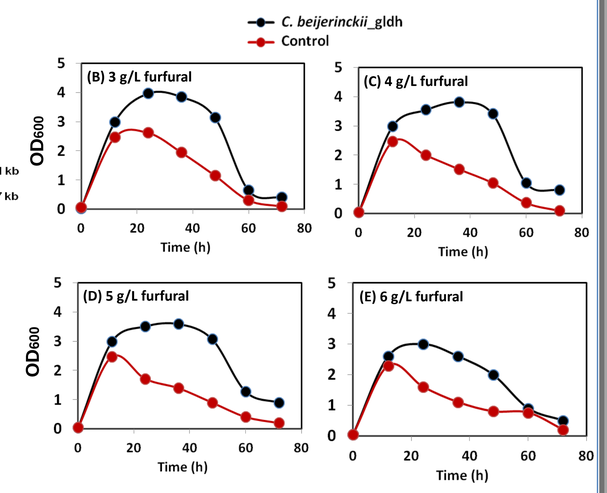


Fig 5: DNA gel image showing PCR amplification of *C. pasteurianum* genes dhaD1, gldA1, dhaK, and generation of fusion constructs **(A)**. Cell growth during fermentation of glucose+glycerol medium by furfural-challenged *C. beijerinckii*_gldh **(B-E)**. At 3, 4, 5, and 6 g/L furfural-challenge, cell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



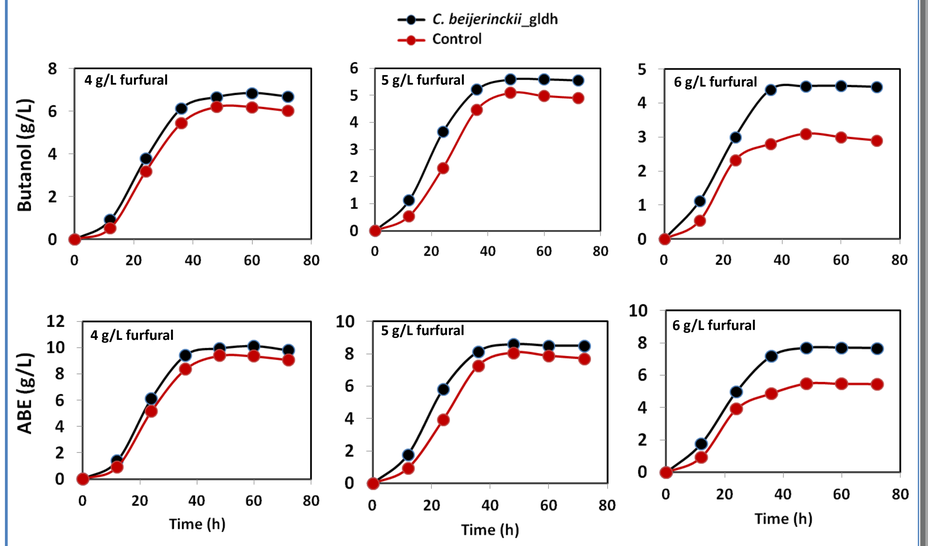


Fig 6: Butanol and ABE profiles during fermentation of glucose+glycerol medium by furfural-challenged *C. beijerinckii*_gldh. Overexpression of two glycerol dehydrogenase genes as a fusion protein increased butanol production by 10% at 4 and 5 g/L furfural challenge and by 46% at 6 g/L furfural challenge. Similarly, ABE production increased to 40% at 6 g/L furfural. The rate 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 in the above-cited 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

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References

❖ Ujor V, Agu CV, Gopalan V and Ezeji TC (2014) Glycerol supplementation of the growth medium enhances in situ detoxification of furfural by *Clostridium beijerinckii* during butanol fermentation. *Appl Microbiol Biotechnol*, 98(14): 6511 – 6521

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